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N° 1

VARIETIES OF THE MENINGOCOCCUS WITH SPECIAL REFERENCE TO A COMPARISON OF STRAINS FROM EPIDEMIC AND SPORADIC SOURCES.

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The following paper is based on the results obtained by cultivating and experimenting with strains of Meningococcus from 45 different cases of Meningitis. Twenty-five of these occurred in Epidemic areas and 20 were Sporadic cases occurring in London. The Epidemic strains are referred to throughout this paper as E. 1, E. 2, E. 3, etc., and the London strains (Sporadic) as L. 1, L. 2, L. 3, etc. For most of the Epidemic strains I am indebted to Dr M. Macdonald of the Lightburn Hospital, Shettlestone, and to Dr C. B. Ker of the Fever Hospital, Edinburgh. Also I have used strains sent to the Lister Institute by Professor Symmers from Belfast and by Dr Buchanan from Glasgow.

The Sporadic strains are all from London sources and were chiefly isolated from specimens sent to the Lister Institute; for other strains I am indebted to Dr Graham Forbes, Dr F. E. Batten and Miss A. Taylor.

The points to which I have specially directed my attention are

(1) the variability of the Meningococcus and the usual directions which variation takes;

(2) the question of a difference between Sporadic and Epidemic strains;

(3) the occurrence in cases of Meningitis of certain other Gram-negative organisms which have some resemblance to the Meningococcus.

VARIATIONS.

The differences observed amongst the strains of Meningococcus which I have cultivated have not concerned the morphology nor the staining reactions.

All the strains have been consistently Gram-negative, and they have never grown in chains. With the one exception of a strain which I described in a previous paper (Arkwright, 1908), no growth has taken place at a temperature of 24° C. It has always been easy to make uniform suspensions of young agar cultures in salt solution (·85 %), and no spontaneous clumping has occurred in these suspensions. On the other hand the fermentation reactions in different sugars have not been quite uniform nor constant.

The sugars chiefly employed have been Glucose, Maltose, Laevulose and Cane sugar.

The fermentation of the sugars was tested in fluid media, either weak broth (25 % peptone broth and 75 % peptone water) or 2 % peptone water, ascitic fluid or serum being added to each tube.

On solid media with sugar and litmus added (method of v. Lingelsheim), the cultures were found to give usually a more rapid, but in many instances a very transient, reaction. The red colour changed again to blue in a few hours and the initial acid reaction varied much in its time of appearance with different strains, so that the results had to be noted at different times for different strains. Trautmann and Fromme (1908) also found that this medium for testing the fermentations of sugars gave irregular results of the same kind.

The reactions with sugar broth were not quite uniform, but were almost always complete by the 5th day.

The most constant features of the fermentations have been the formation of acid from Glucose and Maltose but never from Cane sugar. Laevulose was fermented by several strains, acid being produced when the medium contained broth but never when peptone water only was used. This is interesting, as some English observers have found Laevulose to be fermented, though German writers have usually considered the absence of acid-formation from Laevulose to be an important and distinctive characteristic of the Meningococcus.

With regard to Glucose and Maltose, when broth was used, Maltose was fermented most regularly and sometimes first, but when serum-peptone water only was used the Glucose tube most constantly showed the presence of acid and was generally the first to do so.

Several strains at some stage of their artificial culture lacked the power to produce acid from either Glucose or Maltose or (in a few cases) even from both. For instance Strain L. 5 at the earliest testing fermented no sugars and for months fermented only Maltose, but latterly after 10 months' artificial culture fermented both Glucose and Maltose. Strain

E. 2 on the other hand at first produced acid from both Glucose and Maltose but later fermented neither.

Strains L. 13 and E. 3 never fermented any of the sugars.

The change during long artificial culture in the power of the Meningococcus to ferment some particular sugar has been noted by Trautmann and Fromme (1908). The Meningococcus, therefore, shows evidence of mutation as regards its fermentation characters similar to that recorded by Twort (1907) for the *Bacillus typhosus* under special cultural conditions, and by Andrewes and Horder (1906) to a slight extent for Streptococci.

It may be mentioned here that a strain of Gonococcus, which was isolated from the urethral discharge in a case of acute gonorrhoea in an adult, fermented Glucose and Maltose and not Cane sugar, thus in its sugar reactions resembling most strains of Meningococcus. The fermentation of these two sugars has been found to be characteristic of the Gonococcus by Wollstein (1907) and Gurd (1908). Gordon however found that the Gonococcus fermented Glucose and Galactose but not Maltose and Cane sugar, and this I confirmed for one strain.

Table I shows the sugar reactions given by the various strains of Meningococcus, the Sporadic (L.) and Epidemic strains (E.) being placed in different series.

Table II shows the frequency with which variations from the typical sugar reactions occurred among London and Epidemic strains respectively.

It will be apparent that there is not much difference between the Sporadic and Epidemic strains as regards their sugar reactions; Epidemic strains are rather more uniform than the Sporadic.

SUMMARY OF FERMENTATION TESTS.

When the tests were made in weak broth with the addition of serum, of 14 Sporadic strains 5 fermented Glucose, Maltose and Laevulose, 6 fermented Glucose and Maltose, 1 Glucose and Laevulose, 1 Maltose only, and 1 no sugars. In three of the strains the reactions were not quite constant.

Of 22 Epidemic strains 12 fermented Glucose, Maltose and Laevulose, 9 Glucose and Maltose and 1 no sugars.

When 2 °/₀ peptone water with serum was used as a basis for the sugar media, of 7 Sporadic strains 6 fermented Glucose and Maltose and 1

TABLE I.

	Source			Sugar reactions							
				Broth+serum				2 % peptone water+serum			
No. of sporadic strains (L.)	Cerebro-spinal fluid, meninges or blood	During life or P. M.	Growth at 24° C.	Glucose	Maltose	Laevulose	Saccharose	Glucose	Maltose	Laevulose	Saccharose
1	Men.	P. M.	–	+	+	+	–	+	+	–	–
2	Men.	P. M.	–	+	+		–				
3	C.-S. Fl.	Life	S	+	+		–				
4	Men.	P. M.	–	+	+		–				
5	C.-S. Fl.	Life	–	∓	∓	+	–	∓	∓	–	–
6*	,,	,,	+	–	–	–	–	–	–	–	–
7	,,	,,	–	+	+	+	–				
8	,,	,,	–	+	+	+ s	–				
9 (1)	,,	,,	–	+	+	+	–				
9 (2)*	Blood	P. M.	+	–	–	–	–				
10	C.-S. Fl.	Life	–	+	+	–	–				
11	,,	,,	–	–	+	–	–				
12	,,	,,	–	+	+	–	–				
13	,,	,,	–	–	–	–	–				
14	,,	,,	–	+	+	+	–				
15*	,,	,,	+	–	–	–	–	–	–	–	–
16	,,	,,	–	±	±	±	–	±	±	–	–
17	,,	,,	–								
18	,,	,,	–					+	+	–	–
19	,,	,,	–	+	+	–	–	+	∓	–	–
20	,,	,,	–					+	+	–	–
21	,,	,,	–					+	–	–	–
22	,,	,,	–								
23*	C.-S. Fl.	Life	+	–	–	–	–	–	–	–	
No. of epidemic strains (E.)											
1	C.-S. Fl.	Life	–	+	+	+	–				
2	,,	,,	–	∓	+	+	–				
3	,,	,,	–	–	–	–	–	–	–	–	–
4	,,	,,	–	+	+	+	–	–	–	–	–
5	,,	,,	–	+	+	–	–				
6	,,	,,	–	+	+	∓	–				
7	,,	,,	–	+	+	+	–	+	+	–	–
8	,,	,,	–	+	+		–				
9	,,	,,	–	+	+	+	–				
10	,,	,,	–	+	+	+	–				
11	,,	,,	–	+	+	+	–				
12	Men.	P. M.	–	+	±	±	–	+	+	–	–
13	C.-S. Fl.	Life	–	+	+	–	–				
14	,,	,,	–	+	+	+	–				
15	,,	,,	–	+	+	–	–				
16	,,	,,	–	+	+	–	–				
17	,,	,,	–					+	+	–	–
18	,,	,,	–	+	+	+	–	+	+	–	–
19	Men.	P. M.	–	+	+	–	–	+	∓	–	–
20	C.-S. Fl.	Life	–					+	–	–	–
21	,,	,,	–	+	+	+	–	+	–	–	–
22	,,	,,	–	+	+	–	–	+	–	–	–
23	,,	,,	–	+	+	–	–	+	∓	–	–
24	,,	,,	–					+	+	–	–
25	,,	,,	–	+	+	–	–	+	+	–	–

+ = Acid produced. – = No acid produced.
± = Acid produced on one occasion and not on another.
S = Slight.
* These strains of Gram-negative coccus are not Meningococci.

Glucose only. Two of these strains at another time did not ferment any sugars.

Of 13 Epidemic strains 8 fermented Glucose and Maltose, 3 Glucose only and 2 did not ferment any sugars (*vide* Tables II and III).

Tables II and III show the number of strains giving each variety of fermentation.

TABLE II.

In Serum Broth.

	Glucose, Maltose & Laevulose	Glucose & Maltose	Glucose	Maltose	Glucose & Laevulose	Maltose & Laevulose	No sugars fermented
14 London strains	5	6 (2)	(1)	1 (1)	1	(1)	1
22 Epidemic strains	12	9	—	—	—	—	1

TABLE III.

In Serum Peptone Water.

7 London strains	—	6	1	—	—	—	(2)
13 Epidemic strains	—	8	3 (1)	—	—	—	2

The figures in brackets denote strains which gave different results on other occasions and so appear twice in the Table.

AGGLUTINATION EXPERIMENTS.

The agglutination experiments were all made by the microscopic method at the temperature of the laboratory and the observations were completed at the end of two hours.

Agglutination tests were made with the serum of a horse which was injected with killed, followed by living, cultures of Meningococcus by Dr MacConkey at Elstree. The injections were partly subcutaneous and partly intravenous. At first only one Sporadic strain (L. 1) was used and later after these injections had been made for a year, one strain of Epidemic Meningococcus (E. 12) was injected into the same horse for a further period of 10 months. Serum "1" was obtained by bleeding the horse after injections of strain L. 1 had been carried on for 12 months. This serum agglutinated the homologous strain L. 1 in dilutions up to 1—500, and several other Sporadic strains in dilutions of 1—100, but only three Epidemic strains were agglutinated by it in dilutions higher than 1—5 and none up to 1—100. It was found, however, that five Sporadic strains were likewise not agglutinated by Serum 1 in higher dilutions than 1—5. The strains which agglutinated best were among those which had been longest isolated. As however, at least, one Sporadic strain (L. 8) which had been isolated for as long as one year was among those strains which were not agglutinated, the agglutinability cannot be simply the result of long artificial culture.

TABLE IV.

Microscopic Agglutination.

Serum 1 = Serum of horse injected with Sporadic strain L. 1 only.
Serum 2 = Serum of same horse after receiving in addition the Epidemic strain E. 12 for 10 months.

Date of exp.	Strains of Meningococcus (London strains)	Serum 1					Serum 2					Normal Serum					Normal salt solution	Date of isolation
		1/5	1/25	1/100	1/500	1/2000	1/5	1/25	1/100	1/500	1/2000	1/5	25	1/100	1/500	1/2000		
11/2/08	L. 1	⧻	⧻	⧻	⧺	–	⧻	⧻	⧻	⧺	+	–	–	–	–	–	–	1906
,,	L. 5	⧻	⧻	+	+	–	⧻	⧻	⧻	⧺	+	⧺	+	–	–	–	–	3/4/06
15/2/08	L. 7	⧻	⧻	⧻	+	–	⧻	⧻	⧻	⧺	+		–	–	–	–	–	–/2/07
11/2/08	L. 12	⧻	⧻	⧻	⧺	+	⧻	⧻	⧻	⧻	⧺	⧺	–	–	–	–	–	21/5/07
21/3/08	L. 15*	⧺	–	–	–	–						–	–	–	–	–	–	24/1/08
25/3/08	L. 16	–	–	–	–	–						–	–	–	–	–		28/2/08
21/3/08	L. 18	⧺	–	–	–	–						–	–	–	–	–		11/3/08
,,	L. 19	⧻	–	–	–	–						–	–	–	–	–		17/3/08
,,	L. 20	⧻	+	–	–	–						–	–	–	–	–		–/3/07
26/2/08	L. 21		–	–	–	–						–	–	–	–	–		
	L. 22	⧻	+	–	–	–						⧺	+	+	–	–		
15/2/08	L. 8	⧻	–	–	–	–	⧺	–	–	–	–	+	–	–	–	–		1907
	(Epidemic strains)																	
15/2/08	E. 2	+	–	–	–	–	⧻	–	–	–	–	+	–	–	–	–	–	30/11/06
,,	E. 4	⧻	⧺	–	–	–	⧻	⧻	⧻	+	+	⧺	–	–	–	–	–	18/12/06
14/2/08	E. 5	+	–	–	–	–	⧺	+	+	+s	+s	–	–	–	–	–	–	
,,	E. 7	+	–	–	–	–	⧻	+	–	–	–	–	–	–	–	–	–	8/2/07
11/2/08	E. 12	⧺	–	–	–	–	⧺	+	–	–	–	–	–	–	–	–	–	8/3/07
,,	E. 16	–	–	–	–	–	⧻	–	–	–	–	–	–	–	–	–	–	21/5/07
21/3/08	E. 17	⧻	+		–	–						⧺	+	–	–	–		
26/3/08	E. 18	⧻	⧺	–	–	-						+	–	–	–	–	–	
25/3/08	E. 19	–	–	–	–	–						–	–	–	–	–	–	
26/3/08	E. 21	–	–	–	–	–						–	–	–	–	–	–	
,,	E. 22	–	–	–	–	–						–	–	–	–	–	–	–/3/08
27/3/08	E. 23	–	–	–	–	–						–	–	–	–	–	–	–/2/08
26/3/08	E. 24	–	–	–	–	–						–	–	–	–	–	–	1907
27/3/08	E. 25	–	–	–	–	–						–	–	–	–	–		1907

* This strain of Gram-negative coccus is not Meningococcus.

⧻ = Complete agglutination.
⧺ = Incomplete agglutination.
+ = Slight ,,
+ s = Very slight ,,

The injections of strain E. 12 were carried out on the same horse during the months following the cessation of the strain L. 1 injections. The animal was then bled a second time yielding Serum "2."

This bivalent serum possessed agglutinins for some Epidemic strains, but these could be entirely removed by absorption with the homologous Epidemic strain (E. 12) and partly by absorption with the homologous Sporadic strain (L. 1). The agglutination was only partial however in dilutions above 1—100 for any Epidemic strain.

Serum "3," which came from the same horse at a later date after the injections with E. 12 had been carried on for 10 months, did not give high agglutination with six Epidemic strains, but agglutinated E. 12 (homologous strain) and E. 19 in dilution of 1—500.

Kolle's Anti-Meningococcus serum was used to test the agglutination of 22 strains. This serum agglutinated some Epidemic strains better than any of the Sporadic strains. Of 11 Epidemic strains tested, six were agglutinated in a dilution of the serum of 1—100 or 1—200, whilst of the Sporadic strains tested, only one was agglutinated in a higher dilution than 1—20.

TABLE V.

Microscopic Agglutination.

Serum 3 = Serum of same horse which produced Serums 1 and 2, after it had received injections of Epidemic strain E. 12 for 10 months.

Meningococcus London strains	Serum 3				Normal Serum		Normal salt solution
	1/20	1/100	1/500	1/2000	1/20	1/100	
L. 1	+++	+++	++	+	+++	–	
L. 15*	++	–	–	–	++		
L. 16	–	–	–	–	–		–
L. 19	–	–	–	–	–		–
L. 23*	+++	++	+		+++		++
Epidemic strains							
E. 2	+++	–	–	–	+	–	
E. 7	–	–	–	–	–	–	
E. 12	+++	+++	+++	–	+	–	
E. 18	–	–	–	–	–		–
E. 19	+++	+++	+++	–	–		–
E. 21	–	–	–	–	–		–
E. 22	+++	+	–	–	+		–
E. 23	++	–	–	–	–		–
E. 25	+	–	–	–	–		–

* These strains are not Meningococci.

TABLE VI.

Summary of agglutination tests, showing number of strains agglutinated by different sera in various dilutions. The highest dilution at which each strain agglutinates is considered the dilution appropriate to that strain.

	1/2000	1/500	1/100	1/20	1/5	None
Immune Serum 1.						
11 London strains	—	4	—	2	4	1
14 Epidemic ,,	—	—	—	3	4	7
Immune Serum 2.						
5 London strains	4	—	—	—	1	—
6 Epidemic ,,	1	—	1	2	2	—
Immune Serum 3.						
3 London strains	1	—	—	—	—	2
9 Epidemic ,,	—	2	1	3	—	3
Normal Serum.						
10 London strains	—	—	1	1	3	5
14 Epidemic ,,	—	-	—	1	3	10

Normal horse serum agglutinated one strain (Sporadic) in dilution of 1—100, two strains at 1—20, six at 1—5 and 17 not at all in dilution of 1—5.

Experiments on the power of four Sporadic and two Epidemic strains to absorb agglutinins from Serum 1, showed a power of absorption in the different strains which was to a certain extent proportional to their agglutinability.

Of the Sporadic strains two (L. 1 and L. 5) which were agglutinated about equally and in high dilution (1—500) absorbed their own and each others agglutinins, but absorbed to a much less extent the agglutinins of the other two Sporadic strains.

Strains L. 7 and L. 12 were not agglutinated so well as L. 1 and L. 5 by Serum 1. They were capable of absorbing to a certain extent their own and each other's agglutinins, but hardly removed any of those for L. 1 and L. 5.

The two Epidemic strains, which were only partially agglutinated in dilutions of 1—20 and 1—50, absorbed none of the agglutinins for themselves nor for the other strains.

This experiment then indicates a grouping of these six strains into three groups, viz.:

Group 1. Two Epidemic strains.
Group 2. Two Sporadic strains.
Group 3. Two Sporadic strains of intermediate character.

TABLE VII.

Microscopic agglutination of six strains by Serum 1, before and after it has been absorbed by each strain. In each case 0·25 c.c. of serum after dilution 1/10 was used to emulsify one (24 hour) agar slope of the Meningococcus. The emulsion was incubated at 37° C. for two hours and the cocci removed by centrifugalisation.

Strain of Meningococcus	Serum 1					Serum 1 absorbed by L. 1				
	1/20	1/50	1/100	1/200	1/500	1/20	1/50	1/100	1/200	1/500
L. 1	+++	+++	+++	+++	++	+	–	–	–	–
L. 5	+++	+++	–	–	–	+	+	–	–	–
E. 5	+	+	–	–	–	++	–	–	–	–
E. 7	+	–	–	–	–	+	–	–	–	–
L. 7	+++	+++	+++	++	++	+++	+++	++	–	–
L. 12	+++	++	+	–	–	+	+	+	–	–

	Serum 1					Serum 1 absorbed by L. 5				
L. 1	+++	+++	+++	++	++	–	–	–	–	–
L. 5	+++	+++	+++	+++	++	–	–	–	–	–
L. 7	+++	+++	+++	–	–	+++	+++	++	++	–
L. 12	+++	+++	+++	–	–	+++	+++	+	–	–

	Serum 1					Serum 1 absorbed by E. 5				
L. 1	+++	+++	+++	+++	+	+++	+++	+++	++	++
L. 5	+++	+++	+++	+++	+	+++	+++	+++	+++	++
L. 7	+++	+++	+++	+	+	+++	+++	+++	+	+
L. 12	+++	+++	+++	+++	+	+++	+++	++	–	–

Strain of Meningococcus	Serum 1					Serum 1 absorbed by E. 7				
	1/20	1/50	1/100	1/200	1/500	1/20	1/50	1/100	1/200	1/500
L. 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L. 5	+++		+++	+++	+++	+++	+++	+++	+++	++
E. 5	+	–	–	–	–	+	–	–	–	–
E. 7	–	–	–	–	–	–	–	–	–	–
L. 7	+++		+++	+++	++	+++	+++	++	+	+
L. 12	++	++	+	–	–	++	+	+	–	–

	Serum 1					Serum 1 absorbed by L. 7				
L. 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L. 5	+++	+++	+++	+++	+++	+++		+++	+++	+++
L. 7	+++	+++	+++	+++	+++	+++	++	+	+	+
L. 12	+++	+++	+++	+++	+	+++	–	–	–	–

	Serum 1					Serum 1 absorbed by L. 12				
L. 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
L. 5	+++	+++	+++	+++	++	+++	+++	+++	+++	+
L. 7	+++	+++	++	++	++	+++	+	+	–	–
L. 12	+++	+++	+++	++	+	+++	–	–	–	–

The Epidemic strains E. 5 and E. 7, which are not themselves agglutinated, absorb no agglutinins.

The agglutination experiments shew that numerous injections, extending over a long period, of two strains of Meningococci produced a serum which agglutinated in high dilutions the two homologous and a few other strains, but failed to agglutinate considerably more than half the strains tested.

The experiments also indicate an arrangement of the strains into groups. Each group would consist almost entirely of Epidemic or Sporadic strains. This is shown both by the agglutinations with the sera prepared at the Lister Institute and also by the use of Kolle's serum, assuming that this serum was prepared with strains all of which originated from Epidemic cases.

The variations in the agglutinability which occur among different strains of Meningococcus recorded here have a specific character and are not necessarily correlated with variations in their agglutinability by normal serum.

By the results of absorption experiments with Serum 1 the strains can be arranged into groups, the members of which are apparently closely related whilst having few if any agglutinins in common with the remainder, e.g., a group of the London strains containing L. 1, L. 5, L. 7, and L. 12 of which L. 1 and L. 5 form one sub-group and L. 7 and L. 12 another.

Amongst the Epidemic strains the results of agglutination and absorption closely connect E. 12 and E. 19 whilst shewing no relationship of these to the rest. The agglutination results do not enable us to differentiate accurately the Epidemic strains from the Sporadic strains since there are as great differences between the individual Epidemic and the individual Sporadic strains as between the members of the two groups. For instance, some Epidemic strains were agglutinated (1—25) by Serum 1 (Sporadic) while several Sporadic strains were not agglutinated at all.

The differences between various strains of Meningococci are then very great as regards agglutination. They are not, however, greater than those shown by Torrey (1907) to exist between different strains of Gonococci in respect of agglutination and also of power of fixing complement in the presence of an immune serum (Bordet's method).

FIXATION OF COMPLEMENT (BORDET'S METHOD).

A series of experiments made to test the power of Serum 1 (prepared with one Sporadic strain) to fix complement in a haemolytic system in the presence of different strains of Meningococcus tended, on the whole, to confirm the differences and affinities between the strains as shewn by agglutination.

For these experiments on Fixation of Complement suspensions of agar cultures of Meningococci were used. A trial was made with extracts of the cocci, but the results did not appear to be so satisfactory, perhaps, because certain strains autolysed more readily than others.

The haemolytic serum used was the heated serum of a rabbit which had been immunised with ox corpuscles, and the red corpuscles were those of the ox, washed and suspended in a concentration which corresponded to 2 per cent. of the original blood.

The complement used was that of the guinea-pig diluted 1—10.

0·1 c.c. of the diluted complement was added to 0·3 c.c. of the emulsion of cocci and 0·3 c.c. of Serum 1 (from the horse injected with the Sporadic Meningococcus L. 1). This serum was used in varying dilutions, 1/1, 1/5, 1/25, 1/100 and 1/500.

The degree of correspondence between the particular strain of Meningococcus and the anti-Meningococcic serum was measured by the dilution of serum by which the complement was fixed as indicated by the absence of haemolysis, when the haemolytic serum and corpuscles were added.

The anti-Meningococcic serum in various dilutions, the cocci and the complement were mixed and put in the incubator at 37° C. for 40 minutes. To each tube were then added 1 c.c. of the emulsion of ox corpuscles and 0·1 c.c. of inactivated haemolytic serum diluted 1—10. The whole was then returned to the incubator and left at 37° C. for 1½ hours, after which the tubes were placed at the laboratory temperature for about 20 hours. At the final examination of the tubes it was found that Serum 1 in dilutions of 1—25 had completely prevented haemolysis with some strains of Meningococci, but with other strains the haemolysis had taken place as readily as when cocci or Serum 1 were absent.

The Sporadic Meningococci L. 1, L. 7, L. 12 and L. 8 prevented haemolysis completely or very nearly completely, while L. 5 prevented haemolysis in some experiments, but not in others. These were the only Sporadic strains used. On the contrary, five Epidemic strains did not prevent haemolysis.

A number of controls testing the powers of the individual constituents of the test tubes were made simultaneously and also a control made by substituting normal horse serum for Serum 1. These controls in each instance gave a satisfactory result as shewn in Table IV. When other bacteria were used instead of Meningococci with Serum 1, either haemolysis took place as if no bacteria or serum were present or else the presence of these bacteria prevented haemolysis even in the absence of serum. The bacteria used as controls in this way were Staphylococcus, *Micrococcus catarrhalis* and *B. diphtheriae.*

Summary of Fixation of Complement Experiments.

Of five Sporadic strains of Meningococcus tested with Serum 1 (prepared with one Sporadic strain) one (homologous) strain prevented haemolysis when the serum was diluted to 1—100, three strains when it

TABLE VIII.

Fixation of Complement in a Haemolytic System by different strains of Meningococci in presence of Serum 1.

Complement = Fresh guinea-pig's serum.
H. S. = Rabbit's serum (heated) Immune v. Ox corpuscles.

Complement	Serum 1	Cocci	Time at 37° C. mins.	H. S.	Ox C.	Time at 37° C. hours	Time at 20° C. hours	Haemolysis with different strains										
								No cocci	L. 1	L. 12	E. 5	L. 8	E. 2	E. 4	E. 12	E. 7	L. 7	L. 5
0·1	0·0	0·0	40	0·1	1·0	1½	20	⧻	:	:	:	:	:	:	:	:	:	:
0·1	0·0	0·0	,,	0·0	,,	,,	,,	–	:	:	:	:	:	:	:	:	:	:
0·0	0·0	0·0	,,	0·1	,,	,,	,,	–	:	:	:	:	:	:	:	:	:	:
0·1	0·3	0·0	,,	0·1	,,	,,	,,	⧻	:	:	:	:	:	:	:	:	:	:
0·1	0·3	0·0	,,	0·0	,,	,,	,,	–	:	:	:	:	:	:	:	:	:	:
0·1	0·0	0·3	,,	0·1	,,	,,	,,		⧻	:	:	⧻	:	:	⧻	⧺	⧻	⧻
0·1	0·0	0·3	,,	0·0	,,	,,	,,		–	:	:	–	:	:	:	:	+ sl.	:
*0·1	0·3	0·3	,,	0·1	,,	,,	,,		⧻	⧻	⧻	⧺	⧺	⧻	:	:	⧻	:
*0·1	0·3	0·3	,,	0·0	,,	,,	,,		–	:	:	–	–	–	:	:	–	:
0·1	0·3	0·3	,,	0·1	,,	,,	,,		–	–	–	–	+	+	–	–	–	–
0·1	$0{\cdot}\frac{3}{5}$	0·3	,,	0·1	,,	,,	,,		–	:	:	–	⧺	⧺	⧺	⧺	–	⧺
0·1	$0{\cdot}\frac{3}{25}$	0·3	,,	0·1	,,	,,	,,		–	–	⧻	–	⧻	⧻	⧺	⧺	+ sl.	⧺
0·1	$0{\cdot}\frac{3}{100}$	0·3	,,	0·1	,,	,,	,,		–	:	:	⧺	⧻	⧻	⧺	⧻	+	⧻
0·1	$0{\cdot}\frac{3}{500}$	0·3	,,	0·1	,,	,,	,,		–	:	:	⧻	⧻	⧻	⧺	⧻	+ sl.	⧻

⧻ = Complete haemolysis.
– = No haemolysis.
⧺ = Marked haemolysis.
+ = Slight haemolysis.

* Normal serum used instead of Serum 1 in these tubes.

The dilution 1—25 is the series which best shews difference of strains.

The Serum 1 of horse immunised with one Sporadic strain (L. 1) prevents haemolysis in presence of an emulsion of Strains L. 1, L. 12, L. 8, L. 7 (all Sporadic strains), but not in the presence of E. 2, E. 4, E. 7, and E. 12 (Epidemic strains).

was diluted to 1—25 and one strain gave a variable reaction. Of five Epidemic strains used none did more than partially prevent haemolysis when the dilution was 1—25.

TABLE IX.

Summary of Fixation of Complement Experiments.

	Dilution of Serum	Amount of Serum undiluted c.c.	Emulsions of Meningococcus strains 0·3 c.c. of each									
			L. 1	L. 5	L. 7	L. 8	L. 12	E. 2	E. 4	E. 5	E. 7	E. 12
Anti-Meningococcic	1/5	·06	–	±	–	–	–	⧺	⧺	+	⧺	⧺
Serum 1	1/25	·012	–	±	–	–	–	⧻	⧻	⧻	⧺	⧺
	1/100	·003	–	⧺	+	⧺	+	⧻	⧻	⧻	⧻	⧺
Normal Serum	1/1	·3	⧻	⧻	⧻	⧻	⧻	⧺	⧻	⧻		

⧻ = Complete haemolysis.
⧺ = Marked haemolysis.
+ = Slight haemolysis.
– = No haemolysis; complement completely fixed.

IMPORTANCE OF THE DIFFERENCE BETWEEN STRAINS.

In assessing the value to be put on the differences in sugar fermentation and serum reactions between different strains of Meningococcus, as indications for division of the group into sub-species, the similar variations occurring amongst other groups of bacteria and especially amongst the members of the closely allied species of Gonococcus must be borne in mind.

As mentioned above, Dunn and Gordon (1905) state that the Gonococcus ferments Glucose but not Maltose, whereas Wollstein (1907) and Gurd (1908) found that Glucose and Maltose were both fermented by a large number of strains of Gonococcus. Of two strains of Gonococcus which I isolated from different cases of acute urethritis in male patients the one produced acid from Glucose and Maltose, the other from Glucose but not from Maltose. The sugar reactions of the Gonococcus are therefore not constant.

As regards agglutination, fixation of complement and the formation of antibodies, great similarity has been shown to exist between some strains of Meningococcus and some strains of Gonococcus by Bruckner and Cristéanu (1906), Wollstein (1907), Teague and Torrey (1907), Gurd (1908), and Dopter and Koch (1908).

The differences between the individual races of Gonococcus, or of Gonococcus as tested by these methods, appear to be almost as great as between the two species.

It is, therefore, unlikely that well-marked constant differences along these lines will be found between the groups and sub-divisions of the whole class to which the Gonococcus and Meningococcus both belong.

If specific differences between the two groups of Meningococci exist, other methods must be found than those detailed above by which to demonstrate them. Of the Opsonic test as described by Houston and Rankin (1907) and also used by Taylor (1907) I have no experience.

One feature of the group of Meningococci from Sporadic cases taken as a whole appears to be that its members are more frequently found to deviate from the type to which most strains conform, than is the case with the Epidemic group.

The great variability among the strains shown above points strongly to the desirability of employing a polyvalent serum for therapeutic purposes.

ABERRANT STRAINS OF THE MENINGOCOCCUS, AND ORGANISMS LIABLE TO BE CONFUSED WITH THE MENINGOCOCCUS.

Apart from its morphology the Meningococcus possesses certain characteristics which are almost invariable, viz. (1) its inability to grow at 24° C. and (2) the property which allows a young agar culture to be easily made into a uniform suspension which does not shew spontaneous agglutination.

The other characters are not quite constant and some strains occur which deviate further than usual from the accepted type of the species. For instance, strains L. 13, E. 3 (and E. 4 in peptone water) did not ferment any of the sugars used, though they resembled typical Meningococci in every other way and E. 3 and E. 4 were agglutinated by Kolle's anti-Meningococcic serum.

Another strain L. 5 at one period of its culture fermented no sugars though at a later period it produced acid from Glucose and Maltose. L. 3, obtained in pure culture during life from the cerebrospinal fluid of a case of posterior basic meningitis, gave the usual sugar reactions and agreed with typical Meningococci in every way except that it grew very slowly at 24° C. This strain was mentioned by me in a previous paper (Arkwright, 1907).

Strain L. 9 (2) may be mentioned here though it is not implied that t was a Meningococcus. This strain closely resembled *Micrococcus catarrhalis.* It grew at 20° C., fermented no sugars, resisted attempts to make a uniform suspension of the cocci, clumped spontaneously, and was of characteristic appearance in cultures and microscopically. It was obtained post-mortem from the heart-blood of a case of Sporadic meningitis from the cerebro-spinal fluid of which during life a typical Meningococcus (L. 9 (1)) was isolated.

It remains to mention some interesting micro-organisms which were, perhaps, all of the same species and which were isolated from the cerebro-spinal fluid of three cases of Sporadic meningitis.

These organisms, in stained films, from the cerebro-spinal fluid or from young agar cultures appear as gram-negative cocci which might well be taken for Meningococci. Two of these strains were isolated by Dr W. E. Marshall, to whom I am indebted for them, and one strain was isolated by myself. These strains are denoted in the tables as L. 6, L. 15 and L. 23. One of them (L. 6) was recorded in a former paper as a Gram-negative coccus resembling the description of *M. cinereus* given by v. Lingelsheim (Arkwright, 1907). I think, however, that it probably belongs to the present group. The characters of L. 15 and L. 23 have been more particularly examined and present the following features:—a growth on agar or gelatine at 22° C. is quite distinct in 24 hours; it is transparent and soon becomes confluent. The colonies appear bluish grey by reflected light and yellowish by transmitted light. Glucose, Maltose, Laevulose, and Cane sugar are not fermented. In broth the growth causes uniform turbidity. On MacConkey's bile salt agar there is fair growth. Uniform suspensions of an agar culture in salt solution are easily made and in the case of two strains no spontaneous clumping occurred, but in a suspension of the third strain some clumping occurred without the addition of serum.

Morphology:—A film made from a 24-hours' agar culture and stained by Gram's method with neutral red as a counterstain shews coccal forms in coherent masses or in short lines of 4 or 5. These cocci are rather smaller than most Meningococci, are of uniform size and mostly round, sometimes with an unstained dividing line across the middle. Some double forms like typical Meningococci are present but not in great numbers. In some fields, one or two long bacillary forms are seen which stain rather more deeply with the neutral red than the cocci, and which often shew indications of division into three or four segments. These bacilli are always present though sometimes in very small numbers and are more frequent in older cultures.

In broth cultures the morphology is more that of a short oval bacillus with some long forms.

At first the bacillary forms were regarded as due to a contamination, but microscopical preparations from isolated colonies after plating always presented the same appearance.

Similar organisms were isolated from cases of meningitis by W. James Wilson (1908).

As to the pathogenicity of these Gram-negative organisms and o others more closely resembling *M. catarrhalis*, which have been isolated from the cerebro-spinal fluid or blood in cases of meningitis it would be rash to give too positive an opinion at present. They apparently have no relationship to the Meningococcus of Weichselbaum. They may however be the causal organism in some cases of meningitis, or may play a secondary part.

Conclusions.

1. Whilst most Meningococci produce acid from Glucose and Maltose, strains of undoubted Meningococci are met with which at some stage of their artificial culture ferment only one or neither of these sugars.

2. Other minor differences in the fermentation reactions also occu amongst undoubted Meningococci.

3. These atypical varieties are found amongst strains from both Epidemic and Sporadic cases of meningitis, but more frequently come from Sporadic cases.

4. Specific agglutination in high dilutions (1—500 to 1—2000 obtained with the serum of a horse injected with two strains o Meningococcus was limited to a very few strains besides those used fo the inoculation.

5. Agglutination experiments after the absorption of agglutinin tended to further mark the division of the strains of the Meningococcu used into sub-groups.

6. Experiments on the fixation of complement by the specifi serum in the presence of different strains of Meningococcus confirme on the whole the grouping indicated by the agglutination experiments.

7. The variations observed in the sugar and serum reactions were not such as to indicate a specific difference between the Epidemic and Sporadic strains, for the differences between individual members of each group were as great as any found between the two groups.

8. A Gram-negative coccus resembling more nearly the *Micro-*
occus catarrhalis than the Meningococcus was found in the blood
ost-mortem of a case of Meningococcal meningitis.

9. A Gram-negative bacillus, whose morphology was chiefly that of
micrococcus and closely resembled that of the Meningococcus in the
erebro-spinal fluid and in young cultures, was found in pure or almost
ure culture, in the cerebro-spinal fluid of three cases of Sporadic
eningitis.

I have very much pleasure in recording my thanks to Dr George
ean for the help which he has given me throughout the research, and
o Dr A. T. MacConkey for carrying out the work connected with the
mmunisation of a horse at Elstree.

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ON THE PRODUCTION OF ANTITOXIN BY THE INJECTION OF FILTRATES OF CULTURES OF NON-VIRULENT DIPHTHERIA BACILLI.

By J. A. ARKWRIGHT, M.D.

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We are still in doubt regarding the exact position of those bacilli which, morphologically and culturally, appear to be identical with the Klebs-Loeffler bacillus but which differ from it only in the absence of specific pathogenic power for laboratory animals. Most writers regard such non-virulent strains as true diphtheria bacilli which have lost their virulence and toxigenic power either temporarily or permanently. Others however are inclined to place them in a group allied to that of the genuine Klebs-Loeffler bacillus and express doubts as to whether they at any time possessed a claim to pathological significance (Graham Smith, 1904).

The experiments detailed in this brief communication were made in the course of an attempt to demonstrate the presence of small quantities of specific diphtheria-toxin in broth culture filtrates of these non-virulent strains.

Three non-virulent strains were employed. They were all isolated from the fauces during an investigation of a diphtheria outbreak in a boys' school. Details of the bacteriological findings in connexion with this epidemic have been put on record by the writer (Arkwright, 1908).

Notes regarding the strains employed:

Strain "A" was recovered from the throat of a boy, who two months before had a sore throat and at the time of examination still had a nasal discharge. This strain was of the medium-long type.

Strain "B" was an extremely long and segmented form, while strain "C" was a rather short type. These two latter strains were isolated from boys who had not been observed to suffer from sore throat.

In glucose-peptone water all three strains produced an acid reaction, but cane-sugar media were not altered.

The subcutaneous inoculation of large doses (e.g. 5 c.c.) of 2-day and 8-day old broth cultures, produced no illness in guinea-pigs and, when massive doses large enough to kill were given intraperitoneally, no protection or postponement of death was afforded by the preliminary or simultaneous injection of antitoxin.

Although all attempts to demonstrate by direct methods the presence of toxin in broth culture filtrates of these strains had failed, it was thought that the injection of these filtrates might lead to the development of antitoxin in the horse, even if only very minute quantities of toxin or toxoid were present. Similar experiments by Petrie (1905) were made with strains of Hofmann's bacillus, but with negative results.

Scheme of Inoculation and Serum-tests.

Before the commencement of the inoculations the horse was bled on 4th November 1907 and the normal antitoxin-content of its serum estimated. It was found to possess a quarter of a unit per cub. cent.

Filtrates of 9-day broth cultures of strain "A" were then inoculated in the following quantities at intervals of two or three days: 8th Nov. 1907, 0·5 c.c.; 11th Nov., 1 c.c.; 13th Nov., 5 c.c.; 15th Nov., 10 c.c.; 18th Nov., 30 c.c.; 20th Nov., 80 c.c.; 22nd Nov., 200 c.c.; 25th Nov., 400 c.c.; 27th Nov., 1000 c.c. On Dec. 7th (i.e. 10 days after the last injection) the horse was bled to the amount of six litres.

The serum was now found to contain 4 units per cub. cent.

After a rest, the horse received two large doses of filtrate from strain "B", viz. 24th Jan. 1908, 200 c.c.; and 27th Jan., 600 c.c.

On 11th Feb. 1908 it was again bled when the serum showed an antitoxin value of 25—30 units per cub. cent.

During March 1908 the horse received further injections of filtrate from strain "C" but no appreciable rise in potency resulted.

CONCLUSIONS.

(*a*) Filtered broth cultures of two strains, which were morphologically and culturally indistinguishable from virulent Klebs-Loeffler bacilli but which possessed no pathogenicity for guinea-pigs and apparently were non-toxigenic, led to the development of antitoxin when injected in large quantities into a horse.

One strain raised the potency of the serum from ¼th unit (possessed normally) to 4 units per cub. cent. Further treatment with a second strain caused a further rise to 25 units per cub. cent. Subsequent treatment with a third strain produced no further elevation of the antitoxin-content.

(*b*) These strains though completely non-virulent must be regarded as true *B. diphtheriae* in virtue of their power to excite the production of antitoxin in the horse.

(*c*) Whether these strains have lost their pathogenic properties permanently or only temporarily cannot be stated, but all attempts to raise their virulence or toxigenic power by guinea-pig passage or by cultural methods have so far failed.

Further experiments are in progress on the antitoxin-producing power of various non-virulent strains of *B. diphtheriae.*

To Dr MacConkey I am much indebted for superintending the inoculation of the horse at Elstree.

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Nº 3.

THE PHYSIOLOGICAL EFFECT OF COBALT CARBONYL VAPOUR.

By H. W. ARMIT.

From the Lister Institute of Preventive Medicine.

The discovery of cobalt tetra-carbonyl by Dr Hirtz and Mr Cowapp in Dr Ludwig Mond's laboratories offered a further opportunity of studying the toxic action of volatile compounds of the heavy metals. Through the kindness of Dr Mond, a sufficient quantity of cobalt carbonyl was placed at my disposal for physiological experimentation.

Cobalt carbonyl is a crystalline substance of red colour. It is not very volatile and the vapour has not so characteristic a smell as that of the carbonyls of nickel and iron.

The following experiments were conducted for the purpose of obtaining indications of the volatility as well as of the dissociation of cobalt carbonyl in dry air. Air, freed from carbonic acid by washing in solutions of caustic potash and dried by passing over calcium chloride and through sulphuric acid, was conducted through a tube containing crystals of carbonyl of cobalt kept at a constant temperature by means of a water jacket. The air was then passed through a glass chamber guarded by plugs of cotton wool at the outlet and inlet and thence bubbled through bromine water in a wash bottle and a Meyer's apparatus.

The cobalt found in the bromine water added to that deposited in the plugs and on the walls of the glass chamber corresponded to the amount volatilised, but it was found that, with the current of air employed, saturation was not obtained, unless the temperature of the water jacket was some degrees higher than that of the rest of the apparatus. When volatilised at 0° C. the volume percentage of the carbonyl in a current of air not exceeding 2 litres per minute was very small indeed. The maximum obtained was less than 0·001 volume per cent. At 16° C. the greatest amount of cobalt carbonyl evaporated corresponded to about 0·01 vol. %, but of this 50 % was found as a solid cobalt compound in the wool, etc. It therefore appeared to be unlikely that a mixture of the vapour in air of sufficient concentration to produce acute poisoning in animals would be obtained by such means.

Previous experiments ("The toxicology of nickel carbonyl," *Journ. of Hygiene*, 1907, VII., 4 and 1908, VIII., 5) have shown that at least 15 mgrs. of cobalt per kilogram body weight must be absorbed to kill rabbits. A rabbit weighing two kilograms would therefore require to inhale and absorb approximately 5 c.cm. of cobalt carbonyl vapour. If exposed for two hours at 16° C. to a 0·01 vol. % mixture, 7·2 c.cm. would be contained in the air breathed, but the experiments referred to above show that not less than one half of the quantity of the carbonyl is dissociated, even in the presence of dry air. In the presence of moist air, containing carbonic acid, the amount of dissociation would be greater.

It has been shown that when an animal breathes a mixture of nickel or iron carbonyl and air only part of the vapour breathed is absorbed by the lungs. The expired air, as is the case with other vapour mixtures, contains a certain amount of the vapour. In the case of $Ni(CO)_4$, $Fe(CO)_5$, CO and some other gases, the proportion of vapour absorbed has been found to be about 50 % of the total quantity. It therefore appears that even after two hours' inhalation not more than 1·8 c.cm. of the vapour would be absorbed. Prolongation of the time of exposure to the vapour would favour further dissociation, so that on theoretical considerations, it would seem impossible to produce acute poisoning in an animal with cobalt carbonyl vapour.

In order to test the accuracy of these considerations, rabbits were placed in the glass chamber and allowed to breathe the air mixed with cobalt carbonyl vapour. The temperature of the water jacket was varied in different experiments between 20° and 26° C. The rate of air current was also varied from 0·3 to 2·25 litres per minute. In one experiment, a rabbit was exposed for one hour to the vapour mixture. The cobalt carbonyl was volatilised at 21·5° C. and the air current was kept at 0·3 litre per minute, in order that saturation might be obtained. The temperature of the glass chamber rose from 14·3° to 16° C. during the experiment. After the experiment was over, crystals of cobalt carbonyl were collected from the tube beyond the water jacket, indicating that the air was saturated in the cooler portions of the apparatus. Only 0·175 mgr. of cobalt was recovered from the bromine flask, while 1·2 mgrs. was recovered from the cotton wool plugs. In this case it was impossible to estimate the quantity of cobalt deposited in the chamber itself, as the rabbit's fur would offer as much surface for deposition as the glass walls. On the basis of the blank experiments, it could be estimated that the rabbit had absorbed not more than 0·14 mgr. of cobalt. As was expected, no symptoms of any description followed. Other experiments of a similar nature also yielded entirely negative results.

A rabbit was enclosed in a wooden box provided with glass windows in which was fixed a stretched piece of linen on which crystals of cobalt carbonyl were spread. The rabbit was allowed to remain in the box for two hours. The temperature inside the box rose from 13·5° to 18° C. A slight alteration in the colour of the visible mucous membranes indicated that some carbon monoxide had been absorbed. The amount of this was small. No symptoms of any kind were noted.

From these experiments the conclusion appears justified that owing to its low vapour tension and ready dissociability, cobalt carbonyl, contrasted with nickel and iron carbonyl, is unlikely to produce acute poisoning.

N° 4

ON THE PARATYPHOID AND "FOOD-POISONING" BACILLI, AND ON THE NATURE AND EFFICIENCY OF CERTAIN RAT VIRUSES.[1]

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Part I.—THE DIFFERENTIATION OF THE PARATYPHOID AND "FOOD-POISONING" BACILLI.

The paratyphoid bacilli (A and B) *Bacillus Aertryck*, *B. suipestifer*, *B. Danysz*, *B. enteritidis Gaertner*, and *B. typhi murium* form a closely allied group of organisms; with the exception of *B. paratyphoid* (A), they are morphologically and culturally indistinguishable from one another. A great divergence of opinion, however, exists as to their exact relationship, and although their agglutination reactions serve to classify them into several sub-groups, the classification adopted by different writers is by no means the same.

The importance of determining the relationship of these bacilli lies in the fact that there is ground for believing that some members of the group can produce disease in man, whereas others are stated to be harmless.

In the present investigation an attempt has been made to ascertain how far the various members of this group of bacilli can be differentiated by bacteriological methods.

Description of the Organisms Examined.

1. *B. paratyphoid* (A) (Schottmüller); original strain, 1901.
2. *B. paratyphoid* (B) (Schottmüller); original strain, 1901.
3. *B. Aertryck*; strain isolated from an outbreak of food-poisoning; from Professor v. Ermengem.
4. *B. Danysz*; two strains obtained direct from the Pasteur Institut, Paris.
5. *B. enteritidis Gaertner*; two strains, namely—
    - (*a*) Original strain; from Prof. Gaertner.
    - (*b*) A descendant of the original Gaertner strain; from Dr. Andrewes.
6. *B. typhi murium*; four strains, namely—
    - (*a*) From Professor Löffler.
    - (*b*) From the Pasteur Institut, Paris.
    - (*c*) and (*d*) From Král, Prague.

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[1] Received, February 12, 1909.

7. *B. suipestifer*; two strains, namely—
    (*a*) The laboratory strain (Král),
    (*b*) A strain obtained from Professor Wassermann.

Great pains were taken to obtain genuine and, as far as possible, original strains of the various organisms. As will be seen subsequently, however, several of the strains obtained in the course of this investigation proved on examination either to have been incorrectly identified and labelled, or to consist of a mixture of two bacilli of this group.

METHODS EMPLOYED IN THE INVESTIGATION.

As regards the cultural methods, reference need be made only to a few details.

The observations on the fermentation of the various carbohydrates were made at the end of forty-eight hours after inoculation. The para-dimethyl amidobenzaldehyde test for indol was used. Litmus milk cultures were examined one, three, seven, and fifteen day after inoculation, and usually again some weeks later.

Agglutination tests were invariably made by the macroscopic method; occasionally the microscopic method was used in addition. Instead of broth cultures, emulsions made in normal saline solution from agar cultures grown at 37° C. for twenty hours were used. It is desirable, as Dreyer and Jex-Blake (1906) point out, that the emulsions of the various bacilli used in each experiment should be of approximately the same opalescence. This was attained by making each emulsion of such a density that a series of parallel lines (ruled on paper and kept for the purpose) were just visible through the tubes containing the emulsion; these tubes were of uniform calibre. The results thus obtained are more constant than is the case if the emulsions vary considerably in density.

The mixtures of the bacillary emulsion and the appropriate amount of serum were made up to a constant volume (1 c.c.) with normal saline solution and stirred thoroughly with a fine glass rod before being placed in the incubator.

A reading of the agglutination was taken after incubating the tubes for two hours at 42° C.; they were then kept at room temperature and a second reading was taken on the following morning. The second reading was used mainly as a check upon the first, which is that recorded in most of the results subsequently described. In all cases the highest dilution of the serum at which agglutination was visible to the naked eye, and with the aid of a small hand lens, was taken as the agglutination limit of the serum.

Four degrees of agglutination were noted, namely, + + = complete reaction; + = partial reaction; tr. = very slight reactions; 0 = no reaction. Control tubes containing only emulsion and normal saline solution were made in every experiment. All the sera were monovalent, and were obtained from rabbits, which had received as a rule five or six injections of the vaccine.

Spontaneous agglutination of the emulsions (after incubation) was not infrequent, especially in the case of *B. Aertryck* and *B. paratyphoid* (B). Various methods were tried in the hope of overcoming this difficulty, and the most satisfactory results were obtained by the use of agar cultures grown at 37° C. for twenty hours, these latter being sown from young growths on gelatin.

When the emulsions show a tendency to spontaneous agglutination, the velocity of the reaction is usually much greater in the tubes containing both serum and emulsion than in the control tubes, which contain only the bacterial emulsion. In such cases the first reading of the agglutination limit is apt to be erroneous, and the second reading (taken on the following morning) provides a valuable check against mistakes arising in this way.

The following table illustrates this point:—

TABLE I.—*Serum obtained from a Rabbit immunised with B. Aertryck.*

Dilution of Serum.	*B. Aertryck.* First Experiment.			*B. Aertryck.* Second Experiment.		
	Time of Incubation.			Time of Incubation.		
	One Hour.	Two Hours.	Twenty Hours.	One Hour.	Two Hours.	Twenty Hours.
1–100	+ +	+ +	+ +	+ +	+ +	+ +
1–1,000 . . .	+ +	+ +	+ +	+	+	+
1–2,000 . . .	+ +	+ +	+ +	+	+	+
1–4,000 . . .	+	+	+ +	0	+	+
1–5,000 . . .	+	+	+ +	0	+	+
1–10,000 . . .	0	+	+ +	0	0	0
Control . . .	0	tr.	+ +	0	0	0

Absorption Method.

The organisms used for the absorption of agglutinins were taken from agar cultures not more than forty-eight hours old. The dilution of the serum varied from 1–10 to 1–50, but was usually 1–20 or 1–40; and the dilution of the serum was kept constant, when the relative absorbing power of different bacilli for the serum was being examined.

After the addition of the bacilli the serum was incubated for 1–3 hours at 37° C.; it was then centrifuged, and the clear supernatant fluid was poured off. In some cases the agglutinating power of the serum (after absorption) was determined at one or two definite dilutions; in other cases the limits of agglutination were ascertained.

Since, according to Dreyer, the absorption of agglutinins by bacteria, consists partly in a specific chemical combination of agglutinin and bacteria, and partly in a less specific or non-specific physical absorption, the incomplete saturation of serum with bacilli and the subsequent determination of its agglutination limits would appear to be the more useful method for the differentiation of allied organisms.

RESULTS.

1. *Morphological and Cultural Characters.*

All the members of this group are motile bacilli; from agar cultures the bacilli are often very short and almost coccal in appearance, and show a tendency to bipolar staining with the aniline dyes. They are all decolorised by Gram's method. They neither liquefy gelatin nor form indol.

The particular strain of *B. paratyphoid* (B) used in this investigation grew more vigorously on agar and gelatin, and the growth was moister than was the case with the other organisms; but, according to Kutscher (1906), this difference is inconstant, and many strains of *B. paratyphoid* (B) are culturally indistinguishable from the other members of this group. Their fermentative reactions were as follows:—

Acid and gas were formed from *glucose*, *mannite*, *dulcite*, *lævulose*, *galactose*, *maltose*, and *arabinose*. The amount of gas formed from maltose is very slight, much less than from the other sugars.

No apparent change was observed in *lactose*, *saccharose*, *raffinose*, *salicin*, and *inulin*.

A striking exception to the above statement was met with. One strain of *B. suipestifer* never formed gas in any carbohydrate medium, although it produced acid in the same media as the other strain of this bacillus. This observation was repeated several times at intervals of some weeks, always with the same result. In all other respects these two strains were indistinguishable from one another, and presumably the one strain, while retaining its power of forming acid from various carbohydrates, had never possessed or had lost the capacity to produce gas. A similar strain of *B. suipestifer* has been described by Bock (1906).

B. paratyphoid (A) as a rule produces permanent acidity in *litmus milk*, though occasionally milk may become alkaline after the lapse of several weeks; no clot is formed. The other members of the group produce in *litmus milk* an initial transient acidity, not often lasting more than twenty-four hours; in three to five days, the milk is usually definitely alkaline, and at the end of a week intensely alkaline. After two or three weeks milk often becomes more translucent, but there is no obvious evidence of digestion and no clot is formed.

Attempts have been made to use the time after inoculation at which milk becomes alkaline as a differentiating test between some of these bacilli, but as the time varies considerably even with the same organism on different occasions the test is of no value.

Zupnik (1906) states that in *strongly* alkaline dulcite broth, *B. paratyphoid* (A) forms neither acid nor gas, whereas *B. paratyphoid* (B) still forms acid and gas. I have been unable to confirm this statement.

Löffler's malachite green broth is decolorised in forty-eight hours by all these organisms, with the exception of *B. paratyphoid* (A); at the end of a week, broth inoculated with *B. paratyphoid* (A) was only partially decolorised.

All these bacilli (except *B. paratyphoid*, A) reduce neutral red broth with the production of fluorescence in forty-eight hours. This test, however, gave rather variable results, and the same organism sometimes reduced neutral red broth and at other times failed to do so; as the result of experiment, it appeared that reduction occurred more readily and with greater constancy, when the broth was made slightly more alkaline than usual.

Bahr (1905) has made use of certain culture media which make it possible, in his opinion, not only to differentiate the different members of this group of bacilli, but even to distinguish various strains of *B. enteritidis Gaertner* from one another.

These media are the following:—

1. The salts left after drying and ashing 5 grms. of Cibil's broth (a special brand of meat extract) are dissolved in 500 c.c. of 0·5 per cent. NaCl solution. To this is added 1 per cent. ammonium succinate. According to Bahr, some varieties of *B. Gaertner* can grow in this medium, whereas other strains of *B. Gaertner* cannot grow.

2. To a 1 per cent. solution of Cibil's broth in 0·5 per cent. NaCl solution, is added 0·5 per cent. arabinose; the solution is then made faintly alkaline to litmus. Bahr finds that some varieties of *B. Gaertner* form acid and gas in this medium, others do not.

3. Cibil's broth, 1 per cent.
Sodium chloride, 0·5 per cent.
Racemic acid, 1 per cent.

The racemic acid is exactly neutralised to phenol-phthalein with ammonia; litmus is then added.

Bahr finds that some members of the food-poisoning group of bacilli can act upon the racemate of ammonium, and produce an acid reaction, whereas others fail to do so.

I have examined a number (nine in all) of strains of *B. enteritidis Gaertner* as regards their behaviour in these media. All the strains grew on the first medium, though occasionally one or other of the strains failed to grow; all the strains formed acid and gas from arabinose, and acid from the medium containing the racemic acid.

The results obtained by the use of these media were by no means constant. When examined on different occasions the same organism now formed acid in the racemate broth, and now failed to do so; and in the first medium, growth was not invariable. These media appear, therefore, to be of but little assistance in differentiating the various strains of *B. Gaertner*, which in other respects are indistinguishable from one another.

It was found, in fact, that all the members of this group of bacilli, with the exception of *B. paratyphoid* (A), are morphologically and culturally indistinguishable. This conclusion confirms that reached by Bock, Xylander, Boycott, Morgan, Trautmann, Kutscher and Meinicke and others.

2. *Agglutination Tests.*

All observers, including Bock, Boycott, and Kutscher, appear to be agreed that *B. paratyphoid* (A) stands quite apart from the rest of this group of bacilli in its agglutination reactions.

TABLE II.—*Agglutination Limits for various Organisms.*

Serum.	Author.	*B. Paratyphoid* (B).	*B. Suipestifer.*	*B. Typhi murium.*	*B. Aertryck.*	*B. Gaertner.*
B. paratyphoid (B)	Trautmann .	2,500	...	...	1,000	50
	Bonhoff . .	1,000	...	1,000	...	1,000
	Drigalski . .	2,000	< 400	...	800	400
	Bock . . .	4,000	4,000	4,000	4,000	< 100
	Kutscher and Meinicke .	5,000–15,000	...	15,000–20,000	10,000	< 100
	Böhme . .	20,000	500	< 500	< 100	< 100
	Sacquépée .	1,000–3,000	...	...	100–500	50
	Boycott .	20,000	...	...	20,000	< 100
		5,000	...	...	1,000	200
B. Aertryck	Bock . . .	6,000–7,000	600	6,000	6,000–7,000	< 100
	Kutscher and Meinicke .	2,000	...	2,000	2,000	100
	Böhme . .	100	20,000	...	20,000	< 100
	Trautmann .	500	...	...	5,000	50
	Boycott .	100	...	...	5,000	< 50
		2,000	...	...	2,000	20
	Morgan . .	< 200	...	< 200	1,000	< 200
B. Gaertner.	Trautmann .	100	...	...	100	2,500
	Heller . . .	300	50	...	10,000	100,000
	Boycott . .	100	...	...	50	20,000
B. typhi murium.	Bock . . .	10,000	10,000	10,000	4,000	< 100

The results of agglutination tests are by no means consistent, however, as regards the other members of the groups; and the above table, which is adapted from that given by Sacquépée, summarises the main results obtained by various observers.

While almost all observers find that *B. enteritidis Gaertner* can be distinguished by its agglutination reactions from the other members of the group, the relationship between *B. paratyphoid* (B), *B. Aertryck*, *B. suipestifer*, and *B. typhi murium* is far from clear.

In the present investigation, several strains of certain organisms yielded anomalous agglutination reactions. There can be little doubt that some of these strains were not genuine and had been incorrectly labelled. Two strains supposed to be pure were proved to be mixtures of two organisms.

It seems probable that the divergent results yielded by the agglutination method in the hands of various workers on this group of organisms find at least a partial explanation in the fact that their observations were not always made upon genuine and original strains of bacilli. Some account may be given, therefore, of the anomalous strains met with in the present inquiry.

1. A strain, obtained from the Pasteur Institute, Paris, labelled *B. Danysz*, was identical in its cultural characters and agglutination reactions with *B. paratyphoid* (A). The two organisms were also indistinguishable by absorption tests.

TABLE III.

Serum.	Agglutination Limits of the Organism.			
	B. Paratyphoid (A).	*B. Paratyphoid* (B).	*B. Gaertner.*	Supposed *B. Danysz.*
B. paratyphoid (A) .	50,000	500	100	50,000
Supposed *B. Danysz* .	10,000	100	1000	10,000

Absorption Test.

Serum.	Absorbed with	Agglutination at 1–200 after Absorption.	
		B. Paratyphoid (A).	Supposed *B. Danysz.*
Supposed *B. Danysz* .	Original titre . . .	10,000	10,000
	B. paratyphoid (A). .	0	0
	Supposed *B. Danysz* .	0	0

The organism, alleged to be *B. Danysz*, thus behaved quite differently from other strains of *B. Danysz*; it had almost certainly been incorrectly labelled, and was really a strain of *B. paratyphoid* (B).

2. Four alleged strains of *B. typhi murium* were examined, namely—(*a*) one obtained from Král (Prag); (*b*) obtained through the kindness of Professor Löffler; (*c*) a strain obtained from the Pasteur Institute, Paris, and (*d*) a strain obtained from Král (Prag).

All these strains were identical in their morphological and cultural characters with one another and with *B. Aertryck*. When compared with *B. Aertryck* and *B. enteritidis Gaertner*, their agglutination reactions were as follows:—

Table IV.

Serum.	Agglutination Limits.					
	B. Gaertner.	*B. Aertryck.*	(*a*)	(*b*)	(*c*)	(*d*)
B. Gaertner . . .	10,000	100	10,000	10,000	10,000	100
B Aertryck	< 100	5000	< 100	5,000	< 100	5000
Strain (*a*)	20,000	500	20,000	20,000	20,000	100
,, (*b*)	5,000	4000	...	5,000	5,000	5000
,, (*d*)	500	5000	100	5,000	100	5000

At first sight this result was very confusing, since strains (*a*) and (*c*) resembled *B. Gaertner* in their agglutination reactions, whereas strain (*d*) resembled *B. Aertryck* and the serum of a rabbit vaccinated with strain (*b*) agglutinated equally *B. Gaertner* and *B. Aertryck*. It seemed probable that they were different organisms, and the application of the absorption test showed this to be the case. Strains (*a*) and (*c*) were found to be indistinguishable from *B. Gaertner*.

Table V.

Serum.	Agglutination at 1–200 after Absorption.			
	Absorbed with.	*B. Gaertner.*	Strain (*a*)	Strain (*c*)
B. Gaertner.	Original titre	10,000	10,000	10,000
	B. Gaertner	0	0	0
	Strain (*a*)	0	0	...

The anomalous agglutination reactions of strain (*b*) suggested that possibly it was not a pure culture. It was therefore plated out, and six isolated colonies were picked off and planted on agar. The agglutination reactions of these six subcultures were compared with those of *B. Gaertner* and *B. Aertryck* with the following results :—

TABLE VI.—*Limits of Agglutination.*

Serum.	Isolated Colonies.							
	1.	2.	3.	4.	5.	6.	*B. Gaertner.*	*B. Aertryck.*
B. Gaertner	10,000	< 100	10,000	10,000	10,000	10,000	10,000	< 100
B. Aertryck	< 100	2000	< 100	100	< 100	< 100	< 100	2000

Strain (*b*) was therefore a mixture of *B. Aertryck* and *B. Gaertner.*

Strain (*d*) was indistinguishable by its agglutination reactions from *B. Aertryck* and *B. paratyphoid* (B). But when tested by the absorption method it yielded anomalous results. Partial absorption of a *B. paratyphoid* (B) serum with *B. Aertryck* removed almost all the agglutinins for that organism, leaving the agglutination limits of the serum for *B. paratyphoid* (B) and strain (*d*) almost unaffected (Table VII.).

Similarly, partial absorption of an Aertryck serum with *B. paratyphoid* (B) removed nearly all the agglutinins for *B. paratyphoid*, but left the agglutination limits for *B. paratyphoid* (B) and strain (*d*) almost undiminished.

TABLE VII.—*Absorption Test.*

Dilution of Serum 1–10.

Serum.	Absorbed with	Agglutination Test after Absorption.		
		B. Paratyphoid (B).	*B. Aertryck.*	Strain (*d*).
B. paratyphoid (B)	Original titre . . .	5000	1000	2000
	B. Aertryck . . .	2000 +	< 200	2000
B. Aertryck . . .	Original titre . . .	5000	5000	5000
	B. paratyphoid (B) .	200	2000 +	2000 +

The strain (*d*) thus resembled both *B. Aertryck* and *B. paratyphoid* (B) in its absorption reactions.

Further, a rabbit was immunised with strain (*d*); absorption of its serum with *B. Aertryck* alone or *B. paratyphoid* (B) alone, failed to remove all agglutinins from the serum, although absorption with both these organisms readily removed all agglutinins (Table VIII.).

It appeared possible, therefore, that the strain was really a mixture of *B. Aertryck* and *B. paratyphoid* (B), and this proved to be the case.

The strain (*d*) was plated out, and numerous colonies picked off and grown on agar. The agglutination limits of these strains were tested for (1) a *B.*

TABLE VIII.—*Absorption Test.*

Serum.	Absorbed with	Agglutination at 1-100 after Absorption.		
		B. Aertryck.	*B. Typhi murium* Strain (*d*).	*B. Paratyphoid* (B).
B. typhi murium Strain (*d*) . . .	Original titre . . .	5000	5000	5000
	B. paratyphoid (B) and *B. Aertryck*	0	0	0
	B. Aertryck . . .	0	+	+
	B. paratyphoid (B) .	+	+	0

Aertryck serum after absorption with *B. paratyphoid* (B), and (2) a *B. paratyphoid* (B) serum, which had been absorbed with *B. Aertryck*.

Some of the colonies behaved like *B. Aertryck*, others like *B. paratyphoid*. There can be little doubt, then, that this strain was a mixture of these two organisms.

Consequently it appears that two strains of *B. typhi murium* were indistinguishable from *B. Gaertner*; one was a mixture of *B. Gaertner* and *B. Aertryck*, and one a mixture of *B. Aertryck* and *B. paratyphoid* (B).

In view of these differences, it seems more than doubtful whether the so-called *B. typhi murium* has any independent existence as a recognisable organism.

Excluding these incorrectly labelled organisms, the agglutination method yielded very concordant results, which are exemplified in Table IX. The agglutination limits of *B. Danysz* and *B. Gaertner* were identical for all the sera examined, and these organisms were indistinguishable by this method. Generally speaking, *B. paratyphoid* (B), *B. Aertryck*, and *B. suipestifer* were also indistinguishable by agglutination tests; similar results have been obtained by Kutscher, Bock, and Trautmann. But if a number of rabbits are immunised with the same organism almost all will yield a serum which agglutinates equally these organisms, though one may yield a serum which gives widely differing agglutination limits for these bacilli. The production of such sera depends upon some individual peculiarity in the particular rabbits; and they occur too seldom to invalidate the statement made above, that agglutination tests, as a rule, fail to differentiate these three organisms. There is, however, no difficulty in recognising by agglutination three groups of bacilli, namely:—

1. *B. paratyphoid* (A).
2. *B. Aertryck.* *B. paratyphoid* (B). *B. suipestifer.*
3. *B. Gaertner.* *B. Danysz.*

TABLE IX.—*Agglutination Limits after Incubation at 42° C. for Two Hours, Macroscopic Method.*

In all cases Control Tubes were made, and showed no Agglutination.

Serum.	*B. Paratyphoid* (A).	*B. Paratyphoid* (B).	*B. Aertryck.*	*B. Suipestifer.*	*B. Gaertner.*	*B. Danysz.*
B. paratyphoid (A)	50,000	< 100	< 100	< 100	< 100	< 100
B. paratyphoid (B)	2,000	10,000	...	10,000	< 100	...
,,	...	5,000	...	5,000	< 100	< 100
,,	< 100	5,000	1,000	5,000	< 100	< 100
B. suipestifer	< 100	4,000	5,000	5,000	< 100	< 100
,,	< 100	20,000	...	20,000	< 100	< 100
B. Gaertner	< 20	< 20	...	< 20	50,000	50,000
,,	...	100	...	...	10,000	10,000
,,	< 100	< 100	< 100	< 100	2,000	2,000
B. Danysz	20	< 100	< 100	< 100	5,000	5,000
,,	...	...	...	...	20,000	20,000
B. Aertryck	< 100	5,000	5,000	4,000	< 100	< 100

Absorption Tests.

The absorption method of Castellani was extensively employed in the hope of obtaining a more complete differentiation of these bacilli than is afforded by their agglutination reactions. It was originally devised for the diagnosis of mixed infections, and its value for that purpose is generally admitted. An obvious extension of the principle underlying the absorption method is that if all the agglutinins, formed in response to vaccination with a given organism, are removed from the serum not only by it, but also by some heterologous organism, the two organisms must be identical. Such is the view adopted by Park and Collins, and I have met with no instance in which two bacilli, which were indistinguishable by the absorption test, could be differentiated from one another by any other means. The method promises, therefore, to be of value in differentiating organisms which give identical cultural and agglutination reactions.

Hitherto only a few observations as to its usefulness for the differentiation of the various members of the Aertryck group, or of the Gaertner group, have been recorded, and the results have not been concordant.

Bock succeeded, by this method, in differentiating *B. paratyphoid* (B) from *B. suipestifer* and *B. typhi murium*; and Boycott was able to distinguish *B. paratyphoid* (B) and *B. Aertryck.*

Levy and Fornet (1906) also found that *B. typhi murium* could be distinguished from *B. paratyphoid* (B).

Rieux and Sacquépée (1906), however, and also Vagedes (1905), found that *B. Aertryck* and *B. paratyphoid* (B) were indistinguishable.

Citron (1906) was able to differentiate between *B. suipestifer* and *B. typhi murium.*

In the present investigation the results have been very uniform; their general character is illustrated in subsequent tables.

B. paratyphoid (A) is so readily distinguished from the other bacilli of this group by its cultural and agglutination reactions that very few absorption tests were made upon it.

As regards the Aertryck group of bacilli, there is usually no difficulty in differentiating *B. paratyphoid* (B) from *B. Aertryck* and *B. suipestifer,* whereas the latter two organisms are indistinguishable from one another. The distinction is brought out most clearly by comparing the agglutination limits of the serum for these bacilli after one or more absorptions with a moderate amount of bacilli.

Table X.

Serum.		Agglutination Limit after Absorption.		
		B. paratyphoid (B).	*B. Aertryck.*	*B. Suipestifer.*
B. Aertryck	Original titre	5,000	5,000	...
	Absorbed with— *B. Aertryck*	<200	<200	...
	B. paratyphoid (B)— First absorption	200	4,000	...
	Second absorption	<100	2,000	...
	Third absorption	<100	2,000	...
B. paratyphoid (B)	Original titre	5,000	1,000	1,000
	Absorbed with— *B. paratyphoid* (B)	200	200	200
	B. Aertryck— First absorption	4,000	<200	<200
	Second absorption	2,000	<100	<100
	Third absorption	1,000	<100	<100
B. suipestifer	Original titre	10,000	...	10,000
	Absorbed with— *B. suipestifer*	100	...	100
	B. paratyphoid (B)	<100	...	10,000

Owing to the laboriousness of the method it was not possible to work out the agglutination limits in all cases: and the agglutinating capacity of the serum was usually examined only at one or two dilutions after a fairly complete saturation of the serum with bacilli. The following table illustrates the results which were obtained:—

TABLE XI.

Serum.		Agglutination at 1–200 after Absorption.		
		B. Aertryck.	*B. paratyphoid* (B).	*B. suipestifer.*
B. Aertryck	Original titre	5,000	5,000	2,000
	Absorbed with—			
	B. Aertryck	0	0	0
	B. suipestifer	0	0	0
	B. para-typhoid (B) 1–200	+	0	+
	1–1000	+	0	+
B. paratyphoid (B)	Original titre	5,000	1,000	1,000
	Absorbed with—			
	B. paratyphoid (B)	0	0	0
	B. Aertryck	0	+	0
	B. suipestifer	0	+	0
B. suipestifer	Original titre	5,000	4,000	5,000
	Absorbed with—			
	B. suipestifer	0	0	0
	B. Aertryck	0	0	0
	B. para-typhoid (B) 1–200	+	0	+
	1–2000	+	0	+
B. Aertryck	Original titre	4,000	4,000	4,000
	Absorbed with—			
	B. Aertryck	0	0	0
	B. suipestifer	0	0	0
	B. para-typhoid (B) 1–200	+	0	+
	1–1000	+	0	+
B. paratyphoid (B)	Original titre	...	10,000	10,000
	Absorbed with—			
	B. paratyphoid (B)	...	0	0
	B. suipestifer	...	+	0

It appears, from the preceding tables, that by means of the absorption method *B. paratyphoid* (B) can be differentiated from *B. Aertryck* and *B. suipestifer*, whereas the latter two organisms are indistinguishable from one another.

The inadequacy of the agglutination reaction for the differentiation of *B. paratyphoid* (B) has already been referred to, and at present the absorption test offers the only bacteriological means by which this organism can be identified with certainty.

The fact is of some importance in relation to the bacteriology of "food poisoning." Recently, several outbreaks of "food poisoning" have been recorded in which organisms closely resembling *B. paratyphoid* (B) have been isolated from the fæces or urine of patients, or from the spleen or other organs of fatal cases; and Kutscher (1906) has isolated a similar organism from meat which was the apparent cause of one of these outbreaks. In all these epidemics the incubation period was short (one to two days), the symptoms were those usually observed in food poisoning, namely, vomiting and diarrhœa, and recovery or death occurred in a few days; only one case, observed by Levy and Fornet (1906) at all resembled typhoid fever in its clinical characters.

During one such outbreak, Heller isolated from the spleen of a fatal case a bacillus culturally identical with *B. paratyphoid* (B); its agglutination reactions with monovalent sera, though not very concordant, resembled those of *B. enteritidis Gaertner*. Nevertheless, he named this bacillus *B. paratyphoid* (B), and regarded it as the cause of the outbreak of disease.

In another small outbreak, Levy and Fornet (1906) obtained from the fæces of patients a bacillus culturally identical with *B. paratyphoid* (B); the serum of convalescent patients agglutinated both this bacillus and *B. Aertryck* and standard strains of *B. paratyphoid* (B) in high dilution (200–1000). Similar results were obtained by Kutscher, who, in an outbreak of ninety cases of food poisoning, isolated in nineteen instances a bacillus resembling *B. paratyphoid* (B); the serum of the convalescent patients agglutinated to the same limit of dilution this bacillus and also *B. Aertryck* and known strains of *B. paratyphoid* (B). A monovalent serum, prepared by vaccinating a rabbit with this bacillus, gave similar agglutination reactions. No absorption tests were made. The organism was regarded as being *B. paratyphoid* (B).

From these and other observations (Tiberti and Fromme) the conclusion has been drawn by Kutscher and by Trautmann that *B. paratyphoid* (B) is capable of giving rise to the usual symptoms of food poisoning, namely, acute gastro-enteritis. This conclusion, however, hardly seems to be warranted by the actual evidence. It has already been pointed out that *B. Aertryck* and *B. paratyphoid* (B) are indistinguishable (except occasionally) by their agglutination reactions; and Kutscher and Uhlenhuth consider that they cannot be differentiated in any way from one another.

There seems no reason, therefore, to suppose that the organisms isolated by Levy and Fornet, Kutscher and others were *B. paratyphoid* (B) rather than *B. Aertryck*, since the tests (absorption tests) necessary to the differentiation of these bacilli were not carried out, except for an isolated observation of Levy and Fornet. At present the absorption method furnishes the only means of distinguishing *B. Aertryck* from *B. paratyphoid* (B); and until the bacilli isolated in outbreaks of food poisoning have been examined by this method (in addition to their cultural and agglutination reactions), the possibility that *B. paratyphoid* (B) can give rise to the usual symptoms of food poisoning remains unproved. Meanwhile, so far as can be judged from the available bacteriological evidence, the distinction drawn on clinical grounds between infection by *B. Aertryck* and by *B. paratyphoid* (B) still holds good.

B. Aertryck and *B. suipestifer* are indistinguishable from one another, not only by the bacteriological methods already described, but also, according to Bock and Sacquépée, by their pathogenicity for animals and the morbid changes which they produce. It seems not unreasonable, therefore, to regard them as merely strains of one and the same organism. There are grounds for believing that *B. Aertryck* is pathogenic for man; probably, therefore, the strain known as *B. suipestifer*, is not devoid of risk to man.

B. enteritidis Gaertner and *B. Danysz*, which give identical agglutination reactions with monovalent sera, were also examined by the absorption method, and proved to be indistinguishable from one another.[1]

TABLE XII.

Serum.		Agglutination at 1–100 after Absorption.	
		B. Gaertner.	*B. Danysz.*
B. Gaertner (first rabbit)	Original titre	20,000	20,000
	Absorbed with—		
	B. Gaertner	0	0
	B. Danysz	0	0
		Agglutination at 1–200 after Absorption.	
B. Danysz (first rabbit)	Original titre	20,000	20,000
	Absorbed with—		
	B. Danysz	0	0
	B. Gaertner	0	0
		Agglutination at 1–500 after Absorption.	
B. Danysz (second rabbit)	Original titre	5,000	5,000
	Absorbed with—		
	B. Danysz	0	0
	B. Gaertner	0	0
		Agglutination at 1–500 after Absorption.	
B. Gaertner (second rabbit)	Original titre	10,000	10,000
	Absorbed with—		
	B. Danysz	0	0
	B. Gaertner	0	0
		Agglutination at 1–100 after Absorption.	
B. Gaertner (third rabbit)	Original titre	2,000	2,000
	Absorbed with—		
	B. Danysz	0	0
	B. Gaertner	0	0

Sacquépée has found, also, that they cannot be differentiated by the pathological changes which they cause in animals. These two bacilli, then, appear to be identical with one another.

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[1] No difference between these two organisms was revealed when the agglutination limits of the serum were observed after incomplete saturation with a heterologous bacillus.

Conclusions.

1. The members of the paratyphoid and food-poisoning group of bacilli fall into four sub-groups, namely:—

(1) *B. paratyphoid* (A), which stands alone in both its cultural characters and its agglutination reactions.

(2) *B. paratyphoid* (B), which is indistinguishable from *B. Aertryck* and *B. suipestifer* in its cultural characters and (usually) in its agglutination reactions, but can be differentiated from these two organisms by the absorption method.

(3) *B. Aertryck* and *B. suipestifer*, which cannot be differentiated from one another and which appear to be merely strains of the same micro-organism.

(4) *B. enteritidis Gaertner* and *B. Danysz*, which can be easily distinguished from the preceding sub-groups by their agglutination reactions, but which are indistinguishable from one another, and apparently also are only strains of the same organism.

2. The uniform application of the absorption method is essential for the recognition of *B. paratyphoid* (B), and offers the only means by which it can be identified with certainty.

3. *B. typhi murium* has no existence as a definite organism, since different strains alleged to be *B. typhi murium*, and obtained from accredited sources, were found to differ greatly in their bacteriological characters.

Part II.—ON THE BACTERIAL NATURE AND EFFICIENCY OF CERTAIN RAT VIRUSES.

During the course of the preceding investigation some observations were made on the bacterial contents of certain rat viruses, and upon their efficiency for the destruction of rats.

The following viruses were examined:—

1. Raticide.
2. Ratin No. 1 and No. 2.
3. The Danysz virus.
4. The Liverpool virus.
5. The Ready Rat Relief virus.

The samples of the viruses were bought in the open market, usually from a local chemist. Two or more samples of each virus were procured at an interval of some weeks.

Methods Employed in the Investigation.

1. *Bacteriological Identification of the Organisms contained in the Viruses.*

The same methods were employed in investigating the bacterial contents of the different viruses. A portion of the virus was planted in broth and on agar tubes, and plates were made from the cultures thus obtained. Isolated colonies picked off the plates yielded pure cultures.

The predominating organism obtained from the viruses was a bacillus, and these bacilli were examined as to their morphological and cultural characters, and their reactions on various carbohydrate media.

Observations were also made upon the relation of these organisms to—

1. *B. Aertryck*, a strain obtained from an outbreak of food poisoning (from Prof. v. Ermengen).
2. *B. enteritidis Gaertner*, an original strain (from Prof. Gaertner).
3. *B. Danysz*, two strains obtained from the Pasteur Institute, Paris.

The methods used for this purpose were, first, the cultural characters of these organisms; secondly, their agglutination reactions; and thirdly, their absorption reactions. These methods were employed in the manner already described in detail in the preceding part of this paper, and need not be further referred to.

2. *Methods used for Testing the Efficacy of the Viruses.*

When kept in close confinement, wild rats are apt to die apart from any specific infection, and for this reason experiments made on a small scale and in small cages are quite unsuitable for determining the real efficiency of the viruses; they yield a higher mortality than that obtained when the rats are kept under more favourable conditions. In this inquiry attempts were made to ascertain the destructive power of the viruses for rats living under conditions which approached as closely as possible those of nature, and at the same time allowed accurate observations to be made. The rats had abundance of space, and food and also opportunities for taking shelter when attacked.

The methods were as follows. A cellar was divided into two parts by means of a strong wire-netting, reaching from floor to ceiling and possessing a small door, and the rats were kept in the portion of the cellar closed in by the wire netting; air and light were freely admitted. The cellar was 7½ ft. high, the floor area of the portion enclosed by the netting was 75 square ft., and the available air space was 735 cubic ft. The floor was covered with peat, and two of the walls were lined with broad, wooden shelves. On the shelves and the floor were placed a number of earthenware pipes in which the rats could take shelter.

An abundance of food and water was supplied daily. About a hundred wild rats were used for each experiment, and for this number the space provided was ample. The rats, after being placed in the cellar, were usually kept for a few days, so that any dying from injury during transit might be removed and excluded from the experiment. They were then kept without food for eighteen to twenty-four hours, and the virus, prepared according to the directions (supplied with the virus) was distributed over the cellar. As soon as it had been eaten, food was again supplied to the rats. The number of deaths was noted daily and the dead rats removed from the cellar, post-mortem examinations being made in many cases. At the end of the experiment the surviving rats were killed and counted. The cellar was then cleaned out, the floors, walls, ceiling, and shelves and wire-netting were washed with 2 per cent. lysol, and the drain pipes were plunged bodily into lysol; subsequently the whole cellar was sulphured, and was then ready for a fresh experiment.

These experiments were more favourable to the viruses than would be the case under natural conditions, in that the rats were deprived of food for some hours before and after the bait was placed in the cellar, and that all, or almost all, the bait was eaten. In addition, a few experiments were made on tame white rats kept in small cages in order to ascertain the effects of close confinement.

RESULTS.

1. *Bacterial Contents of the Viruses.*

1. *Cultural characters.*—The Danysz virus, the Liverpool virus, and the Ready Rat Relief virus are issued for sale in the form of cultures on agar slopes; all the samples examined yielded pure cultures of a bacillus. The Ratin viruses (No. 1 and No. 2) consist of a mixture of bread or mashed potatoes and inoculated broth; one sample yielded only a bacillus, two yielded a bacillus and a streptococcus or staphylococcus, and from the fourth sample only a streptococcus was obtained. Raticide is issued in the form of dried flakes. From the first sample a bacillus and a staphylococcus were obtained; from the second sample only *B. coli communis* could be cultivated. The staphylococci, streptococci, and the *B. coli communis* were not further investigated. The bacilli obtained from all the viruses are identical in their morphological and cultural characters. They are short, motile, show some tendency to bipolar staining, and are decolorised by Gram's method. They neither liquefy gelatin nor form indol; they reduce neutral red broth, with the production of fluorescence.

Their reactions to various carbohydrates were as follows:—production of acid and gas occurred in glucose, mannite, dulcite, maltose, galactose, arabinose, lævulose; no obvious change took place in lactose, saccharose, raffinose, inulin, salicin.

Twenty hours after inoculation milk becomes slightly acid; in three to five days it is definitely alkaline, and after seven days strongly alkaline. No clot forms, and there is no obvious change of digestion. All the viruses decolorise Löffler's malachite-green broth in forty-eight hours.

The bacilli obtained from the viruses are indistinguishable by their cultural characters from *B. Aertryck* and *B. enteritidis Gaertner*. Xylander, Trautmann and Mühlens, Dahm and Fürst found that the "Ratin" bacillus was identical in its cultural characters with *B. enteritidis Gaertner*. According to Klein, the bacillus obtained from the Liverpool virus closely resembles *B. enteritidis Gaertner*, although he observed slight cultural differences.

2. *Agglutination Reactions.*

The agglutination reaction of the bacilli obtained from the viruses were compared with those of *B. Aertryck*, *B. Gaertner*, and other organisms. A number of sera were made, and the agglutination limits of the sera for the different bacilli were observed by the macroscopic method, which has already been described (p. 444).

The general result of the observations is indicated in Table XIII. A consideration of this table makes it clear that the Raticide bacillus is indistinguishable from *B. Aertryck*, and that the bacilli obtained from the other viruses are indistinguishable from *B. Gaertner* and *B. Danysz.*

TABLE XIII.—*Limits of Agglutination.*

Serum.	Organism examined.							
	B. Gaertner.	*B. Aertryck.*	Raticide.	Ratin.	Danysz Virus.	Liverpool Virus.	Ready Rat Relief Virus.	*B. Danysz.*
B. enteritidis Gaertner . .	10,000	<100	<100	10,000	10,000	10,000	10,000	10,000
Ratin bacilli .	10,000	...	...	10,000	10,000	10,000	10,000	10,000
Liverpool virus	10,000	<100	<100	10,000	10,000	10,000	...	10,000
B Aertryck .	<100	4,000	4,000	...	<100	<100	<100	<100
B. Danysz .	5,000	<100	...	5,000	5,000	5,000	5,000	5,000
B. Gaertner (second rabbit)	5,000	<100	...	5,000	...	5,000	5,000	5,000

3. *Absorption Reactions.*

Further evidence as to the relationship of these bacilli was obtained by means of the absorption method (*vide* p. 445). The bacilli obtained from Ratin No. 1 and No. 2, from the Liverpool virus and from the Danysz and from the Ready Rat Relief virus were found to be indistinguishable from each other and from *B. enteritidis Gaertner*. The Raticide bacillus was indistinguishable from *B. Aertryck*. The general results of the observations, made by the absorption method, are indicated in Table XIV.

TABLE XIV.—*Absorption Tests.*

Nature of Serum.	Absorbed with	Agglutination Test at 1–500 after Absorption.				
		B. Gaertner.	Ratin.	Liverpool Virus.	Raticide.	*B. Danysz.*
B. Gaertner	*B. Gaertner* .	0	0	0	0	0
	Ratin . . .	0	0	0	...	0
	Liverpool virus	0	0	0	...	0
	B. Danysz . .	0	0	0	...	0
	Original titre .	1–10,000	1–10,000	1–10,000	1–100	1–10,000

Nature of Serum.	Absorbed with	Agglutination Test at 1–500 after Absorption.				
		B. Gaertner.	Ratin.	Liverpool Virus.	Danysz Virus.	*B. Danysz.*
Liverpool virus	*B. Gaertner* .	0	0	0	0	0
	Ratin . . .	0	0	0	0	0
	Liverpool virus	0	0	0	0	0
	B. Danysz . .	0	0	0	0	0
	Original titre .	1–10,000	1–10,000	1–10,000	1–10,000	1–10,000

TABLE XIV. (*continued*).

Nature of Serum.	Absorbed with	Agglutination Test at 1–100 after Absorption.		
		B. Gaertner.	Ratin.	*B. Danysz.*
Rabbit vaccinated with Ratin bacillus	*B. Gaertner*	0	0	0
	Ratin bacillus	0	0	0
	Original titre	1–10,000	1–10,000	1–10,000

Nature of Serum.	Absorbed with	Agglutination Test at 1–500 after Absorption.	
		B. Aertryck.	Raticide.
B. Aertryck	*B. Aertryck*	0	0
	Raticide	0	0
	Original titre	1–200	1–200

II. THE EFFICIENCY OF THE VIRUSES.

Some investigations on the efficiency of "Ratin" No. 1 for destroying rats have been made by Xylander. He found in one experiment that, after being fed three times on the virus, 19 per cent. of the rats died; and the highest mortality ever obtained was 50 per cent., although the rats were not kept under natural conditions. Liston, at the Bombay Bacteriological Laboratory, made a number of experiments, and found that the average mortality caused by feeding rats on Ratin No. 2 was 13 per cent.; the rats were provided with ample space, food, and shelter, and the number fed on the virus was about 200. In the different experiments the death-rate varied from 0 to 40 per cent.

A large number of observations were made by Danysz as to the virulence of the Danysz virus; he found that in 50 per cent. of the experiments all the rats fed on the virus died; in 30 per cent. of the experiments some but not all the rats died; and in 20 per cent. no deaths occurred. Mühlens, Dahm and Fürst, also made some investigations on the efficiency of Danysz virus, and were only able to kill 50 per cent. of grey rats, even when they were kept in cages under ordinary laboratory conditions.

The experiments made in this inquiry were of three kinds. In the first place, a number of experiments were carried out on the destructive power of the viruses for wild rats. Secondly, some observations were made on the efficiency of the viruses for tame rats, kept in small cages and under ordinary laboratory conditions. Thirdly, tests were made as to the agglutinating power of the serum, obtained from rats which survived after being fed on a virus.

1. *Experiments made on Wild Rats in a Cellar.*

In the present inquiry two experiments (except in the case of Raticide) were made as to the efficiency of each of the viruses and of phosphorus. A separate sample of the virus was used for each experiment. All the experiments were carried out under uniform and constant conditions (as regards the method), and the results obtained with the different viruses and with phosphorus are therefore comparable with one another.

The following table summarises the general results of the experiments as to the efficiency of the viruses.

Table XV.—*Experiments made in the Cellar with Wild Rats.*

Virus.	Mortality.	Average of Two Experiments.
	Per cent.	Per cent.
Raticide	21·4	...
Ratin, No. 2—		
First sample	28·4	44·5
Second sample	60·6	
Liverpool virus—		
First sample	42·0	29·6
Second sample	19·3	
Danysz virus—		
First sample	44·0	31·8
Second sample	19·6	
Phosphorus—		
First sample	67·0	64·0
Second sample	61·0	

Experiments made in small Cages with Tame Rats.

Virus.	Mortality.
Liverpool virus	68 per cent.
Danysz virus	63 ,,
Ratin, No. 1	Nil.

The object of the experiments made on tame rats, kept in small cages, was to ascertain the effect of close contact upon the spread of disease from infected to healthy rats.

In all the experiments the same method was employed, and as far as possible the conditions were kept constant. It is unnecessary, therefore, to describe fully the experiments, of which the following is an example :—

Destructive power of the Liverpool virus for rats.

Two tubes of the virus were obtained through a chemist on 13th April 1908. The directions stated that they were efficient until *April* 25. Subcultures made from these tubes yielded the bacillus already described.

April 9.—Ninety-five wild rats were placed in the cellar.

April 13.—The rats, which had been kept without food for eighteen hours, were fed on the virus, prepared according to the instructions. Two hundred and forty baits were scattered throughout the cellar, and when they were eaten the rats again received food.

The duration of the experiment was fourteen days.

Post-mortem examinations revealed congestion of the intestinal mucous membrane and distension of the intestines with gas, enlargement of the spleen, and, in some cases, hæmorrhages, petechiæ or congestion of the lungs.

Cultures were made from the spleen of eight rats; on five occasions bacilli were isolated which were indistinguishable from the bacilli obtained from the virus. Twenty-one rats were partly, and eight completely eaten, except for the skins.

Summary.

Rats placed in cellar	95	
Number found dead	32	total 40
Number entirely eaten (skins only remaining)	8	
Killed at end of experiment	55	

Mortality = 42 per cent.

Agglutination Tests.

The heart blood was obtained from a number of rats which survived after being fed on one or other of the viruses, and examined as to its power to agglutinate the bacillus contained in the virus on which the rats had been fed. In all cases the serum of such rats agglutinated the bacillus in dilutions varying from 1–100 to 1–500, whereas the serum of normal control rats failed to agglutinate at a higher dilution than 1–20.

The presence of agglutinins in the serum of rats, which survived after being fed on a virus although it does not prove conclusively that they were immune to subsequent infection, yet furnishes a strong presumption that the rats had acquired immunity.

SUMMARY.

All the viruses investigated, with the exception of Raticide, owe their efficiency to bacilli which are distinguishable from *B. enteritidis Gaertner*; and the bacillus obtained from Raticide is indistinguishable from *B. Aertryck.*

The wide difference in the mortality caused by the different samples of the same virus indicates the difficulty of maintaining the constancy of its virulence; similar variations have been observed by Xylander and Bahr in the case of Ratin, by Trautmann in the case of *B. enteritidis Gaertner*, by Danysz in the case of *B. Danysz*, and by Liston for Ratin No. 2 and the Danysz virus. It is known, moreover, that spontaneous epidemics among rats, caused by *B. enteritidis Gaertner*, may yield a higher mortality than that brought about by the viruses used in these experiments.

In the present inquiry, for instance, a spontaneous outbreak of disease, proved to be due to *B. Gaertner*, led to the death of 100 per cent. in wild rats; these rats were kept under the same conditions as those obtaining for the experiments.

Under the same conditions, the mortality caused by phosphorus paste was larger than that produced by the viruses, and the effect was more uniform.

The experiments carried out in small cages, in which the rats were closely confined, bring out the influence of unnatural conditions in raising the death-rate, and point to the conclusion that the more nearly the conditions under which the virus is employed approximate to those of nature the lower will be the mortality produced. The extent to which rats were secondarily infected by eating the bodies of those dead of disease was not closely studied in this inquiry. But the mortality was not obviously influenced by the number of rats which were partly or completely devoured in any experiment; and as far as they go these experiments do not indicate that secondary infection plays an important part unless the rats are kept in close confinement.

Finally, there is reason to believe that many rats possess a pre-existent immunity to the viruses, and that others acquire immunity as a result of the infection. Xylander, and also Bahr, found that rats varied greatly in their susceptibilities to "Ratin," but they were unable to correlate the fact with differences in the habitat or food of the rats. Taking into consideration the occurrence among rats of spontaneous epidemics, caused by *B. Gaertner*, the existence of many immune rats is not surprising. Probably, also, rats may acquire immunity as a result of feeding with the virus. In this inquiry the serum of rats which survived after being fed on the virus had a higher agglutinating capacity for the bacilli obtained from the virus than the serum of healthy control rats.

The fact that almost all these rat viruses are stated by the vendors to be harmless to man demands some comment.

As shown above, they all owe their activity to the presence of one or other of two organisms which are indistinguishable from *B. Gaertner* and *B. Aertryck* respectively. Both *B. Gaertner* and *B. Aertryck* were, in the first instance, discovered in connection with outbreaks of food-poisoning in man, and they are generally regarded as the ætiological factor in these outbreaks. Under these circumstances, the statements of the entire innocence of the rat viruses to mankind cannot be accepted without some reserve.

Four separate outbreaks of gastro-enteritis with one death have been observed by Shibayama in Japan, who shows reasonable grounds for attributing them to the careless use of a rat virus containing Löffler's *B. typhi murium*.

Recently, Handson and Williams have reported an outbreak of "food-poisoning" in which they consider the food had become infected by the Liverpool virus. Klein found in the stools of the patients a bacillus which he regards as identical with the Liverpool virus; further, the serum of rabbits infected with the virus agglutinated well both the virus and the bacillus obtained from the patients. The epidemic occurred among persons who took their meals in the room in which the virus had been placed, but no direct evidence was obtained that food had actually been infected by the virus. Danysz has pointed out, however, that although very large amounts of the viruses have been handled during the last few years, without any special precautions, outbreaks of disease in man, traceable to infection by the viruses, are extremely rare. The facts that on the one hand the bacilli contained in the viruses are pathogenic for man, and that on the other hand those who handle and distribute the viruses are very rarely infected, are not easily explained. Reference may be made, however, to two points which seem to bear upon this question. In the first place, the virulence of these bacilli is apparently very variable; and many samples of the viruses do not possess a very high destructive power even for rats. Secondly, it is possible that the importance of *B. Aertryck* and *B. Gaertner* in connection with the causation of "food-poisoning" outbreaks has been overestimated. Mühlens, Dahm, and Fürst have recently brought forward evidence that these organisms may be present in small numbers in apparently healthy meat, apart from any outbreak of disease. The exact relation of these bacilli to food-poisoning calls for further inquiry.

CONCLUSIONS.

1. The viruses examined owe their potency to one or other of two bacilli which are indistinguishable from *B. Aertryck* or *B. enteritidis Gaertner* respectively.

2. Under the conditions of the experiments, namely, the provision for the rats of abundant space, food, and shelter, the destructive power of the viruses was inconstant; the death-rate in different experiments varying from 20 to 50 per cent.

3. Experiments made with phosphorus paste under similar conditions caused a higher and more uniform mortality than did any of the viruses, the death-rate varying from 61 to 67 per cent.

4. There is reason to believe that a certain proportion of the rats fed on the virus become immune, and would therefore be unlikely to succumb to a second infection.

5. The statements of the entire innocence of the viruses for man require justification.

I am indebted to Dr. Andrewes, Dr. Binot, Professor Löffler, and Professor Wassermann for their kindness in supplying me with cultures of certain organisms. I wish to thank Dr. Martin and Professor Dean for frequent suggestions and criticism during this investigation.

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A NOTE ON THE SPIROCHAETE PRESENT IN ULCERATIVE GRANULOMA OF THE PUDENDA OF AUSTRALIAN NATIVES.

By W. CECIL BOSANQUET, M.A., M.D., F.R.C.P.

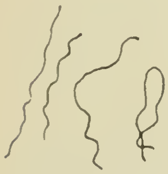
(*From Prof. E. A. Minchin's Laboratory of Protozoology, Lister Institute of Preventive Medicine.*)

(Five Text-figures.)

Early in January of the present year Professor Minchin kindly gave me for examination a portion of tissue and a mounted section taken from a case of the above disease and sent him by Dr J. Burton Cleland, then of Perth, W. Australia. The tissue was already embedded in paraffin, and both it and the section were stained by Levaditi's method and showed the presence of numerous spirochaetes.

These organisms were situated deeply in the tissue, 3 or 4 mm. from the surface, in the fibrous layer forming the base of the ulcer, and were most numerous in the immediate neighbourhood of the blood-vessels of this part. A few were visible within the vessels themselves. The longest forms seen measured on an average 12μ in length: they were so slender that their breadth was rather a matter of guess-work, but it may perhaps be put at $\frac{1}{4}\mu$ (Fig. 1). Shorter forms were common, ranging down to 3 or 4μ in length: these or some of them may have been due to division of longer forms in cutting sections of the tissue. There were a few individual organisms which were somewhat thicker than the average (? $\frac{1}{2}\mu$).

The spirochaetes were in some instances homogeneous throughout in appearance, but a considerable number presented at certain points in their length definite granules staining more deeply than the rest of the protoplasm (Fig. 2). In some cases these granules were distinctly greater in diameter than the rest of the organism. Terminal granules of this latter kind were not uncommon: a few spirochaetes were seen possessing two well-marked granules near to one end (Fig. 3). In some specimens the whole protoplasm was granular throughout, so that the organism resembled a string of beads closely threaded: these were presumably degenerative forms. A few instances of abnormal forms were also seen—*e.g.*, a thick specimen with a well-marked loop or vacuole near to one extremity; a second in which a large terminal granule present was transparent and unstained, giving somewhat the appearance of a loop; and a few individuals coiled up into rings (Fig. 4). I found one or two instances of what appeared to be forked forms, suggesting longitudinal division, and Cleland also saw a few of these; but I do not feel at all certain that these specimens were really bifid, as it is difficult to eliminate possibilities of mere apposition of organisms when dealing with creatures of such tenuity.



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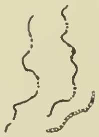

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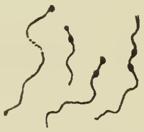

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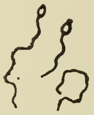

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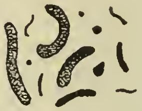

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The curls or waves in the spirochaetes were quite irregular, unlike the symmetrical curls of *Treponema pallidum.* Some organisms lay almost in straight lines: others were twisted on themselves, and others again were merely sinuous (Fig. 1). On the whole they resembled very closely the figures given by Castellani of his *Spirochaeta pertenuis* of yaws, though the illustrations of this parasite given by Levaditi and Nattan-Larrier make it closely resemble *T. pallidum.* In a piece of yaws-granuloma very kindly sent me by Dr Castellani, I was unsuccessful in finding the parasites by the silver-method, as I had hoped to do for the sake of comparison. The spirochaetes in the present case of ulcerative granuloma did not resemble, except in slenderness, the illustrations given by Maclennan of this organism: possibly those from which his figures are taken were derived from the surface of the ulcers and may have assumed a more curved appearance, when thus lying free, than they present when compressed into the interstices of the tissues. No illustrations are given by Wise who first discovered spirochaetes in this disease. He originally found them in smears from the diseased tissues—some of them taken from the deeper parts. He does not, however, speak of having stained the organisms in sections of the tissue.

So far as I am aware, Cleland is the first to supply this proof of the connexion of spirochaetes with the malady.

In the specimens which I examined, the spirochaetes were accompanied by large numbers of bacterial forms. These were found wherever the spirochaetes lay, and were absent where the latter were absent. The bacteria were very irregular in shape and size, ranging from granules about $\frac{1}{2}$—1μ in diameter and slender rods (2 or 3μ by $\frac{1}{2}\mu$) to large rod-shaped organisms, often pretty sharply curved, some 6μ in length and about 1μ in thickness (Fig. 5). The question of the relationship of the bacteria to the spirochaetes is of importance in connexion with that of the aetiology of the disease. Both organisms lay deeply in the tissues and were present in large numbers. The bacteria did not resemble, so far as I could judge, any of the known agents of infective disease: at the same time it seems unlikely that mere saprophytes could establish themselves in such numbers at a point so far from the surface. Hence there is a probability that either the spirochaetes or the bacteria are the causal agents of the ulceration. If this be so, the coexistence of the two forms might be explained either as an example of symbiosis or on the ground that they represent different stages in the development of the same organism. On the latter hypothesis transitional stages might be seen either in the small slender bacterial forms, corresponding as they do with the spirochaetes in thickness and possibly representing the results of transverse division, or in the spherical granules which resemble bacterial spores and may be the granules seen at the extremities and occasionally in the substance of the spirochaetes.

In this connexion it is interesting to recall that in Vincent's angina fusiform bacilli and spirochaetes invariably coexist, and that Ruth Tunnicliffe believed that the latter developed in cultures of the former; that rod-like bodies were found along with spirochaetes on the surfaces of ulcerated cancers by Hoffmann and by Loewenthal; that bacteria and spirochaetes were found together in onychia by Dutton, Todd and Tobey; that Levaditi states that vibrio-forms develop from spirilla in E. African tick-fever; and that Castellani in yaws and Leuriaux and v. Geets in syphilis found oval forms, which the latter authors regard as spores from which the *Treponema* develops. In several of these cases, the question of symbiosis or of varying forms of the same organism is undecided. Markham Carter regards the bacteria present along with the parasites (*Helcosoma tropicum*) in Oriental Sore as symbiotic. The need for such partnership might constitute one of the difficulties in cultivating spirochaetes, which have so far proved practically insuperable. Cleland cultivated bacteria and cocci from the ulcers, but failed to inoculate animals with either of them. He was equally unsuccessful with inoculations of material taken directly from the sore. He has named the spirochaetes of ulcerative granuloma *Spirochaeta aboriginalis* as affecting the aborigines of Australia.

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From the Annals and Magazine of Natural History,
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Notes on the Choanoflagellate Genera Salpingœca *and* Polyœca, *with Description of* Polyœca dumosa, *sp. n.* By J. S. Dunkerly, B.Sc., Assistant in Zoology Department, Birkbeck College, London.

[Plates VI. & VII.]

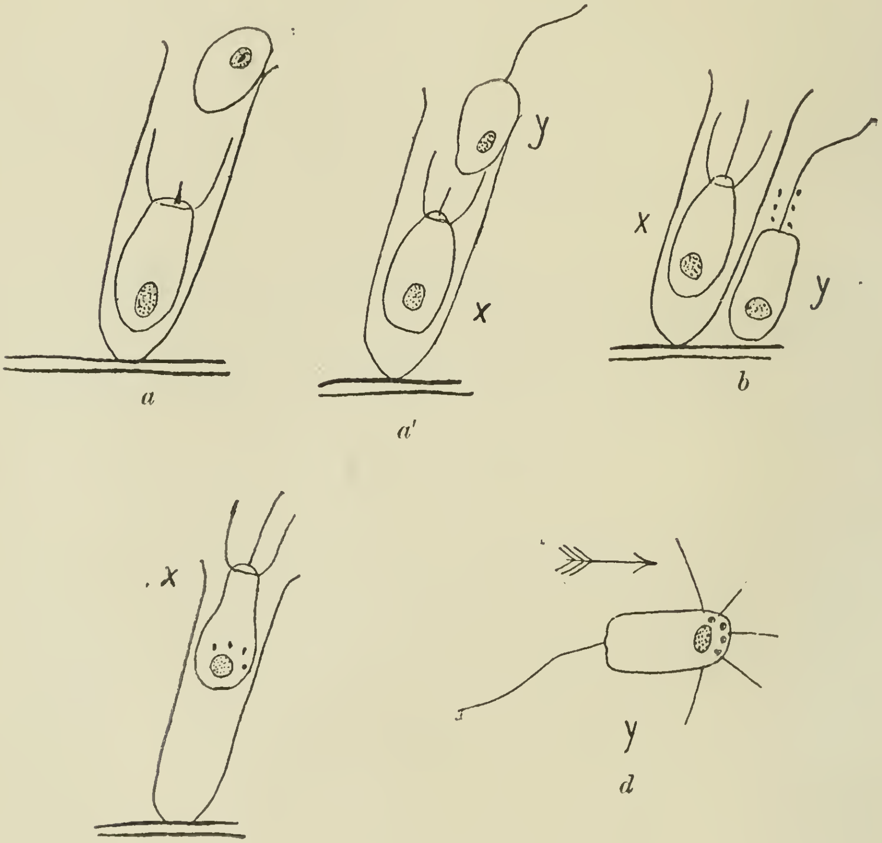
The Choanoflagellata have been little studied of late, and after observing several freshwater forms, I spent a month of 1909 at the Plymouth Biological Station, in order to obtain some knowledge of the marine members of the family. I should like here to express my sincere thanks to the British Association for the Advancement of Science for permission to use their table, and also to the staff of the Laboratory for their kind assistance. My work on these forms has received the kindest encouragement and assistance from Professor Minchin, who has allowed me to work at the Lister Institute.

SALPINGŒCA.

Salpingœca was established as a genus by James Clark (1), and Saville Kent discovered a large number of different forms. That all of Saville Kent's so-called species are truly such is more than doubtful: *e. g.*, Francé has pointed out that Kent's figures of *Monosiga ovata* (2, pl. II. fig. 33) and *M. consociata* (*ibid.* pl. IV. fig. 19) are practically identical. Most of the species of *Salpingœca* admitted by Francé I have seen; one species, the beautiful *S. ampulla*, had not been seen apparently by Francé, the latest monographer of the group.

The commonest species at Plymouth is *S. vaginicola*, which has a very elongate lorica. In this species, which is large, the blepharoplast, which has been seen in *S. amphoridium* by Burck (5), is distinct in preparations stained with carmalum or iron hæmatoxylin (Pl. VI. figs. 1 & 2). Certain interesting results of division were seen (text-fig. 1, *a–d*) in this species. At *a′* the two products of division are seen in the same lorica, and it may be noted that the lower individual (**x**), which at this stage possessed collar and flagellum, is situate in the lower portion of the lorica. The upper individual (**y**),

Fig. 1.



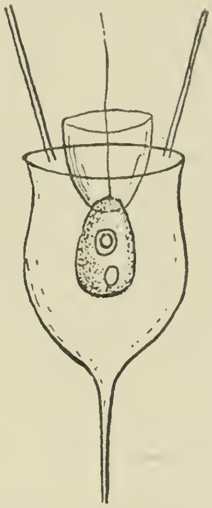
Division (late stages) of *S. vaginicola*. In *d* arrow shows direction of movement. (Diagrammatic.)

which had one flagellum, but no collar, was seen to move slowly away and to settle down as at *b*. After ten minutes it moved slowly away, with its flagellum trailing as a pulsellum (*d*). At this stage **x** had moved up to near the mouth of its lorica, the customary situation (*c*); apparently the animal descends in its lorica for the purpose of division. The free individual (**y**) was observed for an hour to be moving slowly about with feeble movements of its flagellum. Extremely fine pseudopodia were seen at times to be extruded from the aflagellate region of the body (*d*). These may serve as attaching processes when the animal settles down, but the particular individual was unfortunately lost at this stage.

POLYŒCA.

Polyœca dichotoma (text-fig. 2) was the name given to a species of Choanoflagellata found by Saville Kent in a marine tank at the Crystal Palace in 1874. Since that time it has not been recorded, neither Francé (4) nor Bütschli (3) having seen it. Francé admits it as a genus in his monograph (*loc. cit.*), but writes :—" Diese eigentümliche Art, . . . wurde in den letzten 23 Jahren von Niemandem wiedergefunden."

Fig. 2.



Polyœca dichotoma (after Saville Kent).

I found a form obviously answering to Kent's description of this genus quite abundant in a tank at Plymouth Marine Biological Station in August 1909, but have not succeeded in finding it in material from outside.

My drawings and preparations show that, in colony formation and in the shape of lorica, the specimens seen by me differ considerably from those shown in the figures of S. Kent. I am reluctant to establish a new species name in this little-known group, but unfortunately S. Kent's specific name of *dichotoma* would be a positive misnomer for this form. It will be seen (Pl. VI. fig. 2) that from one basal individual as many as five daughter individuals may arise. The form of the lorica is very different from that shown in Kent's figures, the base tapering down very gradually; also the body nearly fills the lorica, whereas Kent shows the body as being about one-half the width of the lorica. The stalk of *Polyœca dichotoma* is shown as branching by Kent. I believe that this was an error of interpretation. Certainly I have never found a stalk branching in my species, although, without careful observation, the crossing of two stalks (Pl. VI. fig. 2) might easily be mistaken for such. Under these circumstances I am compelled to distinguish this species from that of Kent, and, from the bushy form of full-grown colonies, the name *Polyœca dumosa* would appear to be suitable.

The form is very small, the lorica being 10·5 μ in length and 4·5 μ in breadth, while the cell itself is only 6 μ long and 4 μ broad. The flagellum is at least twice as long as the cell-body and is generally in movement along its whole length. The collar is usually very well expanded (Pl. VII. figs. 3 & 4). It is worthy of note that in most of the marine forms seen by me the collar was expanded into the cup-shaped form which Kent always depicts. This is not the case with freshwater forms, or rarely so, the collar being in them more cylindrical in shape. Francé, being more familiar with freshwater forms, was led by this fact to remark that "nur in ganz vereinzelten Fällen ist das Collare tatsächlich glockenförmig," but, in the majority of cases, Kent's figures are probably correct in this respect, as he worked a great deal with marine Choanoflagellata.

The nucleus, not shown in most of Kent's figures of *P. dichotoma*, is seen in preparations stained with carmalum or treated with iodine solution (Pl. VII. figs. 5 & 6). Although very obscure, it can be seen near the base of the flagellum. Like most of the nuclei in Choanoflagellata, it consists of a round evenly staining body, with a central deeply staining karyosome. I have not seen a blepharoplast clearly.

The main interest of this form lies in its faculty for colony formation. Division occurs as described above for *Salpingœca*, the two resulting cells lying somewhat obliquely in the cell (text-fig. 3). The difference between *Salpingœca* and

Polyœca is that the daughter individual in *Polyœca* erects its lorica on the mouth of the mother individual's lorica, instead of swimming away and settling elsewhere.

The excessively attenuate base of the lorica in *Polyœca* leads one to imagine that the length of stalk, in this form at least, is due to the gradual increase in area of that part of the cell which is capable of secreting the material for the formation of the lorica. A diagram (text-fig. 4) shows what may happen in such cases. The successive stages of growth of the cell are shown, and the secreting area in each case is in continuous line, the rest of the cell-outline being dotted. The form of the *P. dumosa* lorica suggests this mode of forma-

Fig. 3.

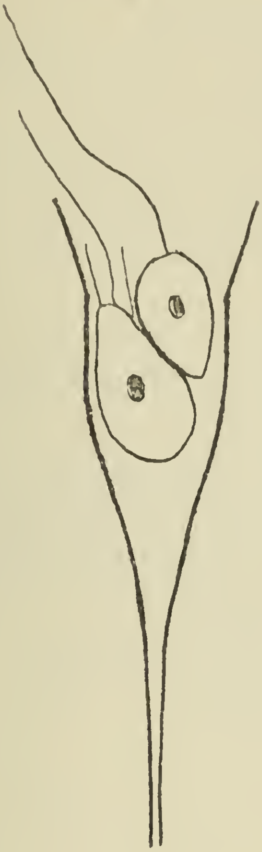


Fig. 4.

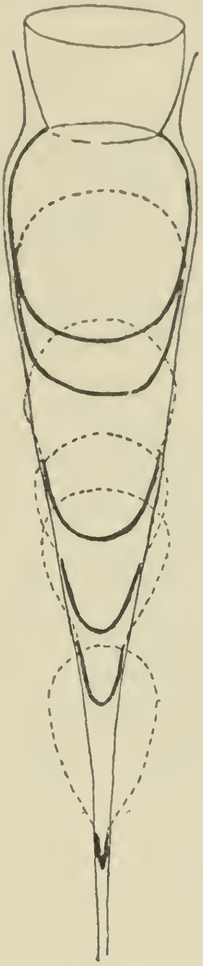


Fig. 3.—Division of *P. dumosa* (late stage).
Fig. 4.—Diagram showing mode of formation of lorica in *P. dumosa*.

tion of the stalk, and on purely *à priori* grounds it would be unlikely for a cell to grow out unprotected on a long stalk, and *then* to form a protective lorica. On the other hand, it is difficult to see how, the lorica being once formed, the stalk could be elongated by a cell situate within the lorica. However, the mode of secretion by similar stalked forms is little understood, but this explanation suggested itself to me after continued comparisons of the younger and older members of a *P. dumosa* colony (Pl. VI. fig. 2, *c* and *d*).

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EXPLANATION OF PLATES VI. & VII.

Fig. 1 *a*. *Salpingœca vaginicola.* Flemming—Fe hæmatoxylin. Individual with long lorica.

Fig. 1 *b*. Ditto. Osmic 4 per cent.—carmalum.

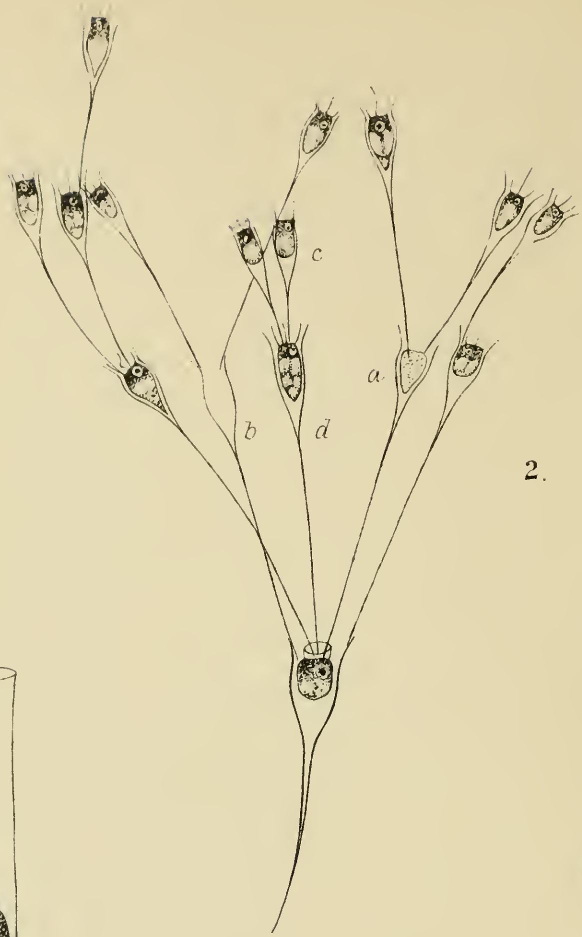
Fig. 2. *Polyœca dumosa*, sp. n. Colony, showing method of growth. *a*, degenerating individual in its lorica; *b*, empty lorica; *c*, young individual with short stalk and narrow lorica; *d*, older individual with longer stalk and wide lorica.

Fig. 3. Single individual of *P. dumosa*, drawn living. Arrows show direction of current of food-vacuoles.

Fig. 4. Colony of *P. dumosa*, drawn living.

Fig. 5. Part of colony of *P. dumosa.* Flemming—carmalum.

Fig. 6. *P. dumosa.* Osmic 4 per cent.—iodine.



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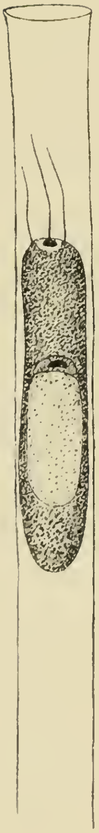

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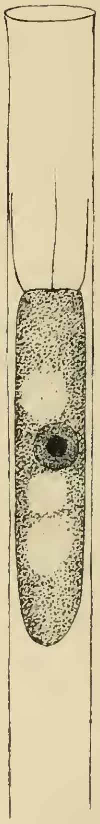

1b.

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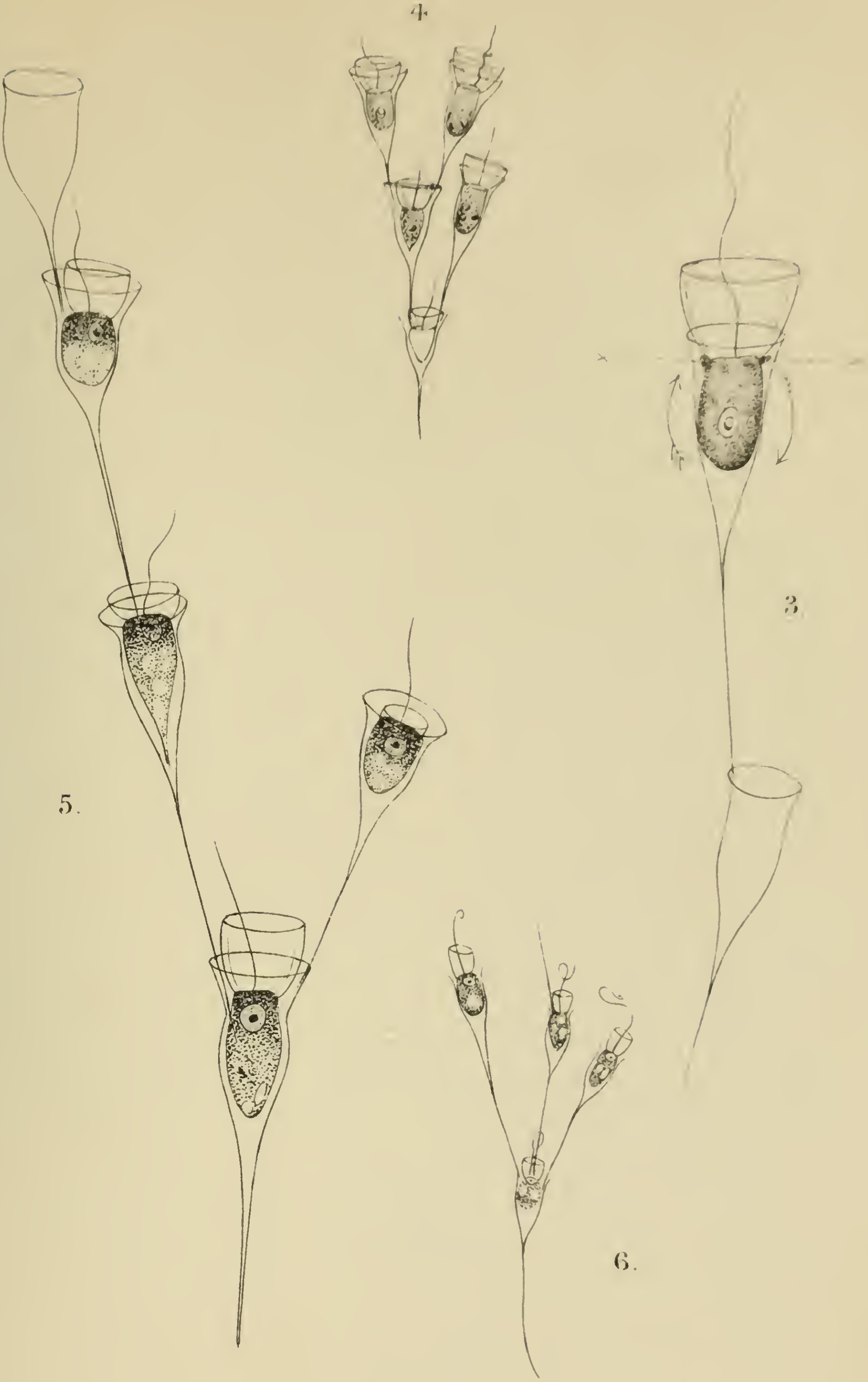

J. Green lith.

[*Reprinted from the* PROCEEDINGS OF THE ROYAL SOCIETY, B. Vol. 81]

The Alcoholic Ferment of Yeast-juice. Part IV.—*The Fermentation of Glucose, Mannose, and Fructose by Yeast-juice.*

By ARTHUR HARDEN, F.R.S., and W. J. YOUNG.

(Received June 10,—Read June 24, 1909.)

The results previously communicated by the authors* were obtained exclusively with glucose, and in the present paper an account is given of the behaviour of mannose and fructose towards yeast-juice both in the presence and absence of added phosphate.

Buchner examined the fermentation of fructose by yeast-juice and found that it proceeded at precisely the same rate as that of glucose.† No experiments with mannose appear to have been previously performed.

The fructose employed throughout these experiments was Kahlbaum's crystallised fructose prepared from inulin. The mannose was prepared by the hydrolysis of ivory-nut and was purified by conversion into the phenylhydrazone, which was recrystallised from hot water and was finally decomposed by benzaldehyde in the usual manner.‡ All the experiments were performed at 25° in the presence of toluene.

I. *Relative Rates of Fermentation of Glucose, Mannose, and Fructose.*

Both mannose and fructose are freely fermented by yeast-juice, as they are also by living yeast. The relative rates of fermentation of these three sugars by yeast-juice vary somewhat in different experiments, but on the average of the experiments performed the fructose appears to be fermented rather more quickly than either mannose or glucose, whilst the mannose is also fermented slightly more rapidly than glucose.

Table I gives the experimental results, 25 c.c. of yeast-juice being employed in each case.

In experiments 1, 2, and 4 the rates were taken after the mixture had been incubated for about half an hour. In experiment 3, observations of rate were made during three different intervals, and the time during which the mixture had been incubated before the commencement of each of these was—(*a*) 25 minutes, (*b*) 70 minutes, (*c*) 130 minutes.

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* 'Roy. Soc. Proc.,' B, 1906, vol. 77, p. 405; 1908, vol. 80, p. 299.

† 'Die Zymasegärung,' p. 100.

‡ Herzfeld, 'Ber. Deutsch. Chem. Ges.,' 1895, vol. 28, p. 440.

Table I.—Relative Rates of Fermentation of Glucose, Mannose, and Fructose by Yeast-juice.

No. of exp.	Amount of sugar.	Total volume.	Time in mins.	Rate of fermentation. Cubic centimetres evolved in the time given.			Ratio of rates.		
				Glucose.	Mannose.	Fructose.	Glucose.	Mannose.	Fructose.
1	1	25·6	45	3·2	3	5·3	1	0·94	1·66
2	1	25·6	60	24·8	28	29	1	1·13	1·17
3*a*	4	45	35	6·8	7·2	8·7	1	1·06	1·28
b	4	45	60	10·4	11	14·1	1	1·06	1·36
c	4	45	265	40·2	38·6	46·9	1	0·96	1·17
4	1·5	32·5	135	51·8	55·9	55·7	1	1·08	1·08

II. *Total Fermentation.*

The total weight of carbon dioxide evolved from an excess of the sugar by a given volume of yeast-juice was also found to be slightly greater with fructose than with glucose, whilst that evolved from mannose in the only two experiments made was considerably less than from glucose.

The following are the experimental results, 25 c.c. of yeast-juice being employed, and the incubation continued until fermentation had ceased:—

Table II.—Total Fermentation of Glucose, Mannose, and Fructose.

No. of exp.	Sugar added.	Total volume.	Total carbon dioxide in grammes.			Ratio of totals.		
			Glucose.	Mannose.	Fructose.	Glucose.	Mannose.	Fructose.
5	4	32·5	0·6556	0·4452	0·7436	1	0·68	1·13
6	4	32·5	0·7405	0·6226	0·8624	1	0·67	1·16

III. *Fermentation of Mannose by Yeast-juice in presence of Phosphate.*

Mannose behaves towards phosphates in the presence of yeast-juice in precisely the same manner as glucose.* A rapid rise in the rate of fermentation occurs; an extra amount of carbon dioxide and alcohol are produced which are equivalent to the phosphate added, and the phosphate is converted into a hexosephosphate which is not precipitable by magnesium citrate mixture and can be isolated in the form of a lead salt. As in the case of glucose, an optimum concentration of phosphate exists at which a maximum rate of fermentation occurs. Beyond this optimum, increase of concentration of phosphate lowers the rate of fermentation. The rates obtained with mannose and glucose in comparative experiments are approximately equal.

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* Harden and Young, *loc. cit.*

These phenomena are illustrated by the following experiments:—

Experiment 7.—Two quantities of 25 c.c. of yeast-juice + 5 c.c. of a solution containing 1 gramme of the sugar were incubated until a constant rate had been attained, and 5 c.c. of an approximately 0·3 molar solution of sodium phosphate were then added.

Experiment 8.—Two quantities of 25 c.c. of a different sample of yeast-juice + 1 gramme of the sugar were incubated as above and 10 c.c. of the same sodium phosphate solution (0·3 molar) were added.

The readings made after the addition of the phosphate are tabulated below, the numbers expressing the volume of carbon dioxide evolved in the five minutes preceding the time given in the first column:—

Table III.—Fermentation of Glucose and Mannose in presence of Phosphate

Time after addition.	Carbon dioxide evolved in preceding 5 minutes.			
	Experiment 7.		Experiment 8.	
	Glucose.	Mannose.	Glucose.	Mannose.
5	4·4	6·4	22·4	22·5
10	8·2	6·0	23·8	26·7
15	9·1	6·8	22·2	22·1
20	9·6	7·0	16·8	14·5
25	7·9	6·0		
30	4·0	4·8		
35	1·5	2·7		
40	1·0	1·0		
45	1·1	1·0		

Experiment 9. *Formation of a Hexosephosphate.*—Twenty-five cubic centimetres of yeast-juice were incubated until the rate of fermentation became constant at 0·8 c.c. in five minutes. Ten cubic centimetres of a 0·3 molar solution of sodium phosphate were then added. The rate rose to 7·1 c.c. in five minutes, and incubation was continued until it had again fallen. The liquid was then boiled and filtered, and the amount of free phosphate estimated as $Mg_2P_2O_7$. The whole solution was found to yield 0·0567 gramme of $Mg_2P_2O_7$. The phosphate added corresponded to 0·3263 gramme $Mg_2P_2O_7$, and hence the difference between these quantities, 0·3263 − 0·0567 = 0·2696, corresponds to the minimum amount of phosphate rendered non-precipitable by magnesium citrate mixture.

Experiment 10. *Equivalence of extra Carbon Dioxide evolved to the*

Phosphate added.—Parallel experiments with glucose, mannose, and fructose were made with yeast-juice from the same preparation, the method being that previously described.* Three quantities of 25 c.c. of yeast-juice + 5 c.c. of a solution containing 1 gramme of the sugar to be examined were incubated with toluene at 25° for one hour, and to each were then added 5 c.c. of a solution of sodium phosphate corresponding to 0·1632 gramme of $Mg_2P_2O_7$ and equivalent to 32·6 c.c. of carbon dioxide at N.T.P. The rates of fermentation were then observed until they had again fallen and attained a steady value, the gases being measured moist at 19°·3 and 760·15 mm.

Table IV.—Relation of Carbon Dioxide evolved to Phosphate added for Glucose, Mannose, and Fructose.

	Glucose.	Mannose.	Fructose.
Maximum attained	9·6	7	11·3
Final rate of juice, cubic centimetres in 5 minutes	1·1	0·96	1·08
Total evolved in 55 minutes after addition of phosphate	49·7	47·8	47·6
Correction for rate of juice in absence of phosphate	12·1	10·6	11·9
Total equivalent to phosphate	37·6	37·2	35·7
" " at N.T.P.	34·4	34·0	32·6

These numbers agree well with the value calculated from the phosphate added, viz., 32·6 c.c.

Experiment 11. *Effect of an Excess of Phosphate.*—Two quantities of 15 c.c. of yeast-juice + 7 c.c. of a solution of mannose containing 1 gramme of the sugar were treated with 10 and 15 c.c. respectively of a 0·6 molar solution of potassium phosphate, K_2HPO_4. The following readings were obtained, showing that in presence of 15 c.c. of the phosphate solution the rate is less than in presence of 10 c.c., and that in neither case is a high maximum rate attained:—

Table V.—Effect of an Excess of Phosphate on the Fermentation of Mannose.

Time after addition in minutes.	Rate in preceding 5 minutes.	
	10 c.c. phosphate.	15 c.c. phosphate.
10	2·2	2·1
20	1·9	1·6
30	2·4	1·4
40	3·0	1·5
60	4·2	1·7

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* 'Roy. Soc. Proc.,' B, 1906, vol. 77, p. 414.

IV. *Fermentation of Fructose by Yeast-juice in the presence of Phosphate.*

Fructose, like mannose, agrees qualitatively with glucose in its behaviour towards phosphates, but it differs quantitatively from both these sugars in two important respects: (1) The optimum concentration of phosphate is much greater; (2) the maximum rate of fermentation attainable is much higher.

These points of resemblance and dissimilarity are brought out by the following experiments:—

(*a*) When a phosphate is added to yeast-juice containing fructose the rate of fermentation rises to a maximum and then falls to a rate which is usually slightly higher than the original rate of fermentation.

Experiment 12.—Ten cubic centimetres of a 0·3 molar solution of sodium phosphate were added to a mixture of 25 c.c. of yeast-juice and 1 gramme of fructose, the original rate of fermentation of which was 0·8 c.c. in five minutes. The total volume of the mixture was 35·6 c.c. The readings were as follows:—

Table VI.—Fermentation of Fructose in presence of Phosphate.

Time after addition of phosphate in minutes.	Cubic centimetre of CO_2 evolved in preceding 5 minutes.
5	14·9
10	21·3
15	21·7
20	17·3
25	10·9
30	2·0
35	1·5
40	1·0
45	1·2

The maximum rate attained varies very considerably with different samples of yeast-juice, as is shown by the following numbers (see Table VII), which refer in each case to 25 c.c. of yeast-juice.

It is interesting to note that the two high rates, 80 and 76·2 c.c. per five minutes, are equal to about half the rate obtainable with an amount of living yeast corresponding to 25 c.c. of yeast-juice, assuming that about 40 grammes of yeast are required to yield this amount of juice, and that this amount of yeast would give about 140 c.c. of carbon dioxide per five minutes at 25°, which has been found experimentally to be the average rate obtainable with the top yeast employed for these experiments.

Table VII.—Maxima attained by the Fermentation of Fructose in presence of Phosphate.

Number of experiment.	Volume of 0·6 molar phosphate solution added.	Total volume.	Maximum rate attained, cubic centimetres CO_2 in 5 minutes.
	c.c.	c.c.	
13	12 ·5	75	80
14	12 ·5	50	27 ·1
15	10	50	31 ·2
16	20	55	76 ·2

(*b*) Equivalence of the extra carbon dioxide evolved to the phosphate added. One example of this has already been given in Experiment 10, p. 338.

Experiment 17.—In another case the phosphate added was equivalent to 65·2 c.c. of CO_2 at N.T.P.

	c.c.
Gas evolved in 45 minutes	82 ·2
Correction for rate of juice 1 ·2 c.c. per 5 minutes for a period of 45 minutes	10 ·8
Gas evolved at 19 and 747 ·75	71 ·4
Volume at N.T.P.	64 ·2

(*c*) Production of a hexosephosphate non-precipitable by magnesium citrate mixture.

Experiment 18.—The experiment was carried out precisely as Experiment 9. The amounts of phosphate are expressed as $Mg_2P_2O_7$:—

Phosphate added	0 ·3263
Free phosphate after fermentation	0 ·0426
Phosphate rendered non-precipitable	0 ·2837

The solution after boiling was found to contain a hexosephosphate which has been isolated in the form of a lead salt and is at present undergoing investigation.

(*d*) Existence of an optimum concentration of phosphate.

The following table shows the maximum rates produced by the addition of varying volumes of a 0·6 molar solution of potassium phosphate, K_2HPO_4, to yeast-juice and fructose. In all comparable experiments the total volumes were kept equal by the addition of a solution of potassium bicarbonate as previously explained for glucose;* in each case 2 grammes of fructose were employed. The maximum obtained and the optimum concentration are printed in thick type:—

Table VIII.—Maximum Rates of Fermentation and Optimum Concentrations of Phosphate for Fructose.

No. of experiment.	Volume of yeast-juice.	Total volume.	Cubic centimetres of 0·6 molar solution of K_2HPO_4 added.	Maximum rate per 5 minutes.
	c.c.	c.c.		c.c.
19*a*	5	25	0	0·5
b	5	25	**5**	**14·2**
c	5	25	10	5·5
d	5	25	15	1·8
20*a*	15	40	3	22·5
b	15	40	**7·5**	**25·4**
c	15	40	10	20·7
d	15	40	15	11·3
e	15	40	20	7·4
21*a*	10	35	3	31·5
b	10	35	**5**	**32·2**
c	10	35	7·5	28·5
d	10	35	10	20·2
e	10	35	15	9·2
f	10	35	20	5·7

It thus appears that, precisely as in the case of glucose, progressive increase in the concentration of phosphate beyond the optimum produces a corresponding decrease in the rate of fermentation, and at a high concentration the rate becomes extremely slow.

(*e*) Comparison of the optimum concentrations of phosphate and of the maximum rates produced at those concentrations for fructose and glucose.

The following results, which all refer to 10 c.c. of yeast-juice, clearly show that the optimum concentration of phosphate for the fermentation of fructose is from 1·5 to 10 times that for glucose, and that the maximum rate of fermentation for fructose is two to six times that of glucose.

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* 'Roy. Soc. Proc.,' B, 1908, vol. 80, p. 307.

Table IX.—Optimum Concentrations of Phosphate and Maximum Rates of Fermentation for Fructose and Glucose.

No. of experiment.	Sugar, in grammes.	Total volume.	Optimum volume of 0·6 molar phosphate.		Maximum rate in cubic centimetres of CO_2 per 5 minutes.	
			Glucose.	Fructose.	Glucose.	Fructose.
22	2	35	2	5	7·5	32·2
23	4	50	1	10	5·4	28·4
24	1·6	23	2	5	8	17
25	1	25	1·75	5	5·2	25·9
26	2	25	5	7·5	16·2	31·2
27	2	20	2	3·5	7·9	22·6
28	2	22·5	0·75	2	3·4	22·2

V. *Effect of the Addition of Fructose on the Fermentation of Glucose or Mannose in presence of a large Excess of Phosphate.*

When the rate of fermentation of glucose or mannose by yeast-juice is greatly lowered by the presence of a large excess of phosphate, the addition of a relatively small amount of fructose causes rapid fermentation to occur. This induced activity is not due solely to the fermentation of the added fructose, since the amount of this sugar may be insufficient to yield the gas evolved.

The general nature of this phenomenon may be gathered from the following experiment:—

Experiment 29.—Two quantities of 25 c.c. of yeast-juice + 2 grammes glucose + 20 c.c. of 0·6 molar K_2HPO_4 solution + toluene were incubated at 25°. The amount of phosphate was largely in excess of the optimum, and the rate of fermentation was found to be 1·8 c.c. per five minutes.

A. To one of these were added 1 c.c. of glucose solution containing 0·2 gramme of the sugar, and 4 c.c. of the phosphate solution. The rate of fermentation fell to 1·5 and continued at this value.

B. To the other were added 1 c.c. of a solution containing 0·2 gramme of fructose and 4 c.c. of the phosphate solution. The rate of fermentation at once rose, as shown by the following readings.

As the evolution of carbon dioxide proceeds the phosphate is converted into hexosephosphate, and its effect on the fermentation of the glucose lessened, and hence in order to maintain the original concentration of phosphate it is necessary to add a fresh quantity at intervals to B, the amount required being calculated from the gas evolved, 13·5 c.c. of CO_2 at

Time after addition.	CO_2 evolved in preceding 5 minutes.	
	Substances added.	
	A. Glucose and phosphate.	B. Fructose and phosphate.
5 minutes	1·5	2·6
10 ,,	1·8	6·9
15 ,,	1·2	13·8
20 ,,	1·5	19·4
25 ,,	1·5	25·1

17°·2 and 762·4 mm. being equivalent to 2 c.c. of the phosphate solution. The further course of the experiment was as follows:—

B.

Time after addition of phosphate.	Gas evolved since last addition of phosphate.	Phosphate added 0·6 molar solution.	Rate per 5 minutes.
minutes.	c.c.	c.c.	c.c.
25	67·8	4	25·1
30	—	—	27·1
35	53·9	10	26·8
40	—	—	22·1
45	—	—	19·7
50	—	—	18·5
55	—	—	19·8
60	—	—	20·5
65	120·5	10	19·9
70	—	—	17·3
75	—	—	18·2
80	—	—	17·4
85	—	—	15·8
90	—	—	16·8
95	97·9	—	12·4
Total gas evolved	340·1		

Hence, although the concentration of the phosphate was never allowed to fall much below the original value and was generally considerably above it, the addition of 0·2 gramme of fructose to 2 grammes of glucose produced a total evolution of 340·1 c.c. of CO_2, corresponding to the total fermentation of 1·3 grammes of sugar (in the ratio $C_6H_{12}O_6 : 2\ CO_2$), whereas in the absence of fructose the total fermentation would have been only 28·5 c.c., corresponding to 0·22 gramme of sugar. As the fructose added was only 0·2 gramme and the subsequent evolution of carbon dioxide corresponded to the total fermentation of 1·3 grammes of sugar, it is obvious that the addition of the fructose must have induced the fermentation of the glucose.

Experiment 30.—Similar results were obtained in another way by employing so large an excess of phosphate that the fermentation observed did not reduce the concentration of phosphate to the limit at which the rapid fermentation of glucose in the absence of added fructose became possible.

Four quantities of 15 c.c. of yeast-juice + 5 c.c. of a solution containing 2 grammes of glucose were employed—

1. 5 c.c. of 0·6 molar potassium phosphate solution were added.
2. 7·5 c.c. of phosphate solution were added.
3. 15 c.c. of phosphate solution were added.
4. 15 c.c. of phosphate solution and 0·5 c.c. of a solution containing 0·05 gramme of fructose were added.

The following observations were then made :—

Time.	Rate for 5 minutes.			
	1.	2.	3.	4.
5 minutes	2·0	1·9	0·8	4·2
10 „	2·6	1·6	1·1	4·0
15 „	3·7	2·2	0·6	6·6
20 „	3·7	2·1	0·8	8·9
25 „	5·2	2·6	0·7	11·6
30 „	6·4	2·9	0·6	14·2
35 „	9·6	3·5	0·7	14·8
Total evolved	33·2	16·8	5·3	64·3

In this case the amount of gas evolved in No. 4 is equivalent only to 4·8 c.c. of the phosphate solution, and the final concentration is therefore 10·2 c.c. of phosphate in 35 c.c. The concentration of phosphate, therefore, never falls as low as that present in No. 2 (7·5 in 27·5 or 9·5 in 35), and yet the fermentation is much more rapid than in this flask, which itself contains a concentration of phosphate greatly in excess of the optimum, as shown by a comparison with No. 1. The amount of carbon dioxide yielded by the complete fermentation of 0·05 gramme of fructose is only 13·5 c.c., so that there can be no doubt that most of the gas evolved was derived from the glucose.

(Note.—Experiments 1, 2, and 4 are not strictly comparable, since the contents of the flasks were not made up to the same volume, but the difference in rate due to this is negligible.)

VI. *Specific Character of the Inductive Action of Fructose.*

This inductive effect is specific to fructose and is not produced when glucose is added to mannose or fructose, or by mannose when added to glucose or fructose, under the proper conditions of concentration of phosphate in each case.

The experiments on this point were carried out by ascertaining in each case the excess of phosphate necessary to produce a slow rate of fermentation and then making two parallel experiments, one with, and the other without, the addition of the small quantity of the sugar to be tested for inductive power. All the observations were made with 15 c.c. of yeast-juice.

Table X.—Specific Character of the Inductive Effect of Fructose.

No. of experiment.	Cubic centimetres 0·6 molar phosphate.	Total volume.	Grammes of sugar present.			Gas evolved.	Time.
			Mannose.	Fructose.	Glucose.		
		c.c.				c.c.	mins.
31*a*	15	37	1	0	0	8·7	30
b	15	37	1	0·1	0	43·3	30
c	15	37	1	0	0·1	10·9	30
32*a*	10	32	1	0	0	18·9	30
b	10	32	1	0·1	0	76·3	30
33*a*	60	82·5	0	1	0	13·6	20
b	60	82·5	0·15	1	0	7·9	20
34*a*	10	30	0	0	1	11·4	30
b	10	30	0·1	0	1	14·1	30
35*a*	75	90	0	2	0	18·4	30
b	75	90	0	2	0·1	16·1	30

This remarkable property of fructose, taken in connection with the facts that this sugar in presence of phosphate is much more rapidly fermented than glucose or mannose, and that the optimum concentration of phosphate for fructose is much higher than for glucose or mannose, appears to indicate that fructose when added to yeast-juice does not merely act as a substance to be fermented, but, in addition, bears some specific relation to the fermenting complex.

All the phenomena observed are, indeed, consistent with the supposition that fructose actually forms a permanent part of the fermenting complex, and that, when the concentration of this sugar in the yeast-juice is increased, a greater quantity of the complex is formed. As the result of this increase in the concentration of the active catalytic agent, the yeast-juice would be capable of bringing about the reaction with sugar in presence of phosphate at a higher rate, and at the same time the optimum concentration of phosphate would become greater, exactly as is observed. The question whether, as suggested above, fructose actually forms part of the fermenting complex, and the further questions, whether, if so, it is an essential constituent, or whether it can be replaced by glucose or mannose with formation of a less active complex, remain at present undecided, and cannot profitably be more fully discussed until the results of experiments now in progress are available.

It must, moreover, be remembered that different samples of yeast-juice vary to a considerable extent in their relative behaviour to glucose and fructose, so that the phenomena under discussion may be expected to vary with the nature and past history of the yeast employed.

Summary.

1. Mannose behaves towards yeast-juice both in the presence and in the absence of added phosphates in the same manner as glucose.

2. Fructose resembles both glucose and mannose in its behaviour towards yeast-juice, but in the presence of phosphates is much more rapidly fermented than the other sugars, and the optimum concentration of phosphate is much higher.

3. Fructose has the property of inducing rapid fermentation in presence of yeast-juice in solutions of glucose and mannose containing such an excess of phosphate that fermentation is only proceeding very slowly. No similar property is possessed by glucose or mannose.

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The Alcoholic Ferment of Yeast-juice. Part V.—*The Function of Phosphates in Alcoholic Fermentation.*

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Two equations have been proposed by the authors* to represent the course of alcoholic fermentation by yeast-juice:

(1) $2C_6H_{12}O_6 + 2PO_4HR_2 = 2CO_2 + 2C_2H_6O + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2$,
(2) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HR_2$.

These were founded on (*a*) the determination of the amount of carbon dioxide and alcohol produced by the addition of a known amount of phosphate in presence of excess of sugar†; (*b*) the production of a hexosephosphate of the composition $C_6H_{10}O_4\ (PO_4R_2)_2$; (*c*) the occurrence of an enzymic hydrolysis of this substance with production of free phosphate.

In order to obtain further experimental justification for this view, several additional determinations have been made, and these form the subject of the present paper.

I. *Ratio of Carbon Dioxide produced to Sugar fermented in Presence of Excess of Phosphate.*

When glucose or fructose is fermented by yeast-juice in presence of excess of phosphate, a period of accelerated fermentation occurs, during which two molecules of carbon dioxide are evolved and one molecule of hexosephosphate formed from two molecules of sugar added. The ratio between sugar added and carbon dioxide evolved is determined by adding a known weight of sugar, together with an excess of sodium phosphate to yeast-juice at 25°. The phenomena then observed are precisely similar to those which occur when a phosphate is added to a fermenting mixture of yeast-juice and excess of sugar, as previously described (*loc. cit.*). The rate of fermentation rapidly rises, and then gradually falls until a rate is attained approximately equal to that of the auto-fermentation of the juice in presence of phosphate. At this point it is found that the extra carbon dioxide evolved, beyond the amount which would have been given off in the absence of added sugar, is equivalent to only half the sugar according to the equation:

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_6O,$$

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* 'Roy. Soc. Proc.,' B, 1908, vol. 80, p. 299.

† 'Roy. Soc. Proc.,' B, 1906, vol. 77, p. 405.

but exactly equivalent to the sugar according to the authors' equation (1) as given above.

Experiments 1 *and* 2.—Two quantities of 25 c.c. of yeast-juice were incubated at 25° for 15 minutes.

1. To one was added 4 c.c. of a 0·6 molar solution of dipotassium hydrogen phosphate, saturated with carbon dioxide, and 1 c.c. of a solution containing 0·2 gramme of fructose (pure from inulin). The volumes of gas evolved in successive periods of 5 minutes were then read. As soon as a steady rate had been attained, a second addition of 4 c.c. of potassium phosphate solution and 1 c.c. of fructose solution was made, and the volumes of gas evolved observed as before.

2. The second portion of yeast-juice was treated in the same way, except that 0·2 gramme of pure glucose was substituted for the fructose.

The following are the actual readings :—

Time after addition.	Fructose.		Glucose.	
	First addition.	Second addition.	First addition.	Second addition.
	c.c.	c.c.	c.c.	c.c.
5	20 ·6	20 ·5	6 ·9	17 ·0
10	9 ·2	8 ·2	9 ·7	
15	4 ·2	4 ·7	12 ·0	12 ·8
20	2 ·8	3 ·6	6 ·2	5 ·4
25	2 ·4	2 ·6	3 ·0	3 ·3
30	1 ·9	2 ·6	2 ·3	2 ·6
35	2 ·2	2 ·1	1 ·8	2 ·2
Total	43 ·3	44 ·3	41 ·9	43 ·3
Fermentation of juice at 2·2 c.c. per 5 mins.	15 ·4	15 ·4	15 ·4	15 ·4
Extra carbon dioxide ...	27 ·9	28 ·9	26 ·5	27 ·9

The gas was measured moist at 17° and 762·4 mm.

The volume calculated at the same temperature and pressure—

(*a*) From the ratio $C_6H_{12}O_6 : 2CO_2 = 53·92$
(*b*) „ $2C_6H_{12}O_6 : 2CO_2 = 26·96$.

II. *Fermentation in Presence of Small Amounts of Phosphate.*

Numerous attempts have been made to procure conclusive experimental evidence that alcoholic fermentation cannot take place in the absence of phosphates. Hitherto, however, it has not been found possible to free the materials employed completely from phosphorus compounds which yield phosphates by enzymic hydrolysis during the experiment, but it has been found that when the phosphate contents are reduced to as low a limit as possible, the amount of sugar fermented becomes correspondingly small, and, further, that in these circumstances the addition of a small amount of phosphate produces a relatively large increase in the fermenting power of the enzyme.

It was previously shown that the addition of phosphate to a mixture of yeast-juice and a sugar not only caused the evolution of an equivalent amount of carbon dioxide, but, in addition, produced an increase in the total fermentation amounting to from 10 to 150 per cent. of the original.

It now appears that when the total phosphorus present is largely reduced, the increase produced by the addition of a small amount of phosphate may amount to as much as 700 per cent. of the original, in addition to the quantity equivalent to the phosphate, whilst the actual total evolved, including this equivalent, may be as much as twenty times the original fermentation. This result must be regarded as strong evidence in favour of the view that phosphates are indispensable for alcoholic fermentation.

The results indicated above were experimentally obtained in three different ways. In the first place (Experiments 3 and 4) advantage was taken of the fact that the residues obtained by filtering yeast-juice through a Martin gelatin filter are sometimes found to be almost free from mineral phosphates, whilst they still contain a small amount of coferment. The experiment then consists in comparing the fermentation produced by such a residue poor in phosphate with that observed when a small amount of phosphate is added. The second method (Experiment 5) consisted in carrying out two parallel fermentations by means of a residue rendered inactive by filtration and a solution of coferment free from phosphate and hexosephosphate (prepared by a method shortly to be described) and adding a small amount of phosphate to one of the solutions.

The third method (Experiment 6) consisted in washing zymin with water to remove soluble phosphates and then adding to it a solution of coferment containing only a small amount of phosphate, and ascertaining the effect upon the fermentation of the addition of a small known amount of phosphate.

Experiment 3.—A slightly active filtration-residue was employed, 1 gramme of which contained a total amount of phosphorus corresponding with 0·038 gramme of $Mg_2P_2O_7$.

a. 1 gramme residue + 2 grammes fructose + 20 c.c. water.
b. The same mixture + 0·26 gramme crystallised sodium phosphate.

Experiment 4.—A second slightly active residue was used, the total phosphorus in which was not estimated.

a. 1 gramme residue + 2 grammes fructose + 20 c.c. water.
b. The same mixture + 0·25 gramme crystallised sodium phosphate.

Experiment 5.—In this case an inactive filtration-residue containing no free phosphate and total phosphorus equivalent to 0·0342 gramme $Mg_2P_2O_7$, and a solution of coferment free from mineral phosphate and hexosephosphate, but containing total phosphorus equivalent to 0·077 gramme $Mg_2P_2O_7$ per 25 c.c., were used.

a. 1 gramme residue + 2 grammes fructose + 25 c.c. coferment solution.
b. The same + 0·25 gramme crystallised sodium phosphate.

Experiment 6.—*a.* 25 c.c. washed zymin suspension + 15 c.c. coferment solution + 2 grammes fructose.

b. The same mixture + 2·5 c.c. of a 0·3 molar solution of sodium phosphate solution.

All the experiments were carried out at 25°, in presence of toluene, and the results are tabulated below.

	Experiment			
	3.	4.	5.	6.
	c.c.	c.c.	c.c.	c.c.
Gas evolved in absence of added phosphate ...	1·4	1·2	20·3	31·2
Gas evolved in presence of added phosphate ...	25·8	26·8	92·3	187·2
Increase due to phosphate	24·4	25·6	72·0	156
Carbonic acid equivalent to phosphate...........	16·9	16·8	16·8	19
Increase after initial period...........	7·5	8·8	55·2	137
Ratio of totals	18·4	21·3	4·5	6
Ratio of increase after initial period to original fermentation	5·3	7·3	2·7	4·4

III. *Hydrolysis of Hexosephosphate by Yeast-juice with Production of Free Phosphate and a Fermentable Sugar.*

(*a*) Production of free phosphate and a reducing substance.

It has previously been shown that free phosphate is produced by the action of the enzymes of yeast-juice on the hexosephosphate formed during fermentation.* According to equation (2) above, this phosphate should be accompanied by a sugar, and the following experiment shows that this is actually the case :—

Experiment 7.—Potassium hexosephosphate was incubated with an inactive filtration-residue, so that no fermentation of any liberated hexose could occur, and the amounts of free phosphate and reducing substance formed were determined, allowance being made for the quantities produced by the incubation of the residue and of the hexosephosphate separately.

5 grammes of an inactive filtration-residue were dissolved in water and made to 75 c.c., and a solution of potassium hexosephosphate, free from glucose and free phosphate, and

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* Harden and Young, 'Roy. Soc. Proc.,' B, 1908, vol. 80, p. 299.

containing phosphorus equivalent to 0·2352 gramme $Mg_2P_2O_7$ in 25 c.c., was prepared. The following mixtures were then made and incubated at 25° in presence of toluene:—

(1) 25 c.c. residue + 25 c.c. water.
(2) 25 c.c. hexosephosphate + 25 c.c. water.
(3) 25 c.c. residue + 25 c.c. hexosephosphate.

After 5 hours' incubation the solutions were heated for 5 minutes in boiling water, made up to a known volume, and the phosphate and reducing power estimated in aliquot portions of the filtrate. In order to ascertain the reducing power of the product, it was necessary to remove the unaltered hexosephosphate, and this was effected by precipitation with basic lead acetate, the estimation of the reducing power being made by means of Pavy's ammoniacal copper solution. In the following table the amounts of phosphate are expressed as grammes of $Mg_2P_2O_7$, and the reducing power as grammes of glucose in the quantities originally incubated.

Solutions.	Free phosphate as $Mg_2P_2O_7$.			Reducing substance as glucose.		
	Before.	After.	Produced.	Before.	After.	Produced.
1	0	0·0141	0·0141	0	0	0
2	0·0009	0·0030	0·0021	0·0244	0·0275	0·0031
3	0·0009	0·0968	0·0959	0·0244	0·0595	0·0351

The amount of reducing substance expressed as glucose produced by enzymic hydrolysis is therefore $0{\cdot}0351 - 0{\cdot}0031 = 0{\cdot}032$, and that of phosphate is $0{\cdot}0959 - (0{\cdot}0141 + 0{\cdot}0021) = 0{\cdot}0818$ gramme.

(*b*) Proof that the reducing substance is a sugar.

Experiment 8.—50 grammes of zymin (Schroder) were washed in the centrifuge four times with water, in order to remove as much as possible of the coferment and thus to diminish the fermenting power of the zymin, and the residue was made to 120 c.c. with water. The following solutions were then incubated, Nos. 2 and 3 being added to ascertain the amount of sugar formed by the spontaneous hydrolysis of the hexosephosphate and from the carbohydrates of the zymin:—

1. 50 c.c. washed zymin suspension + 55 c.c. of a solution of sodium hexosephosphate, equal to 19·9 c.c. normal solution, and equivalent if completely fermented to 223 c.c. of carbon dioxide at N.T.P.
2. 50 c.c. washed zymin suspension + 55 c.c. water.
3. 50 c.c. water + 55 c.c. sodium hexosephosphate solution.

No. 1 gave a total evolution of 25·3 c.c. carbon dioxide under atmospheric conditions, whilst Nos. 2 and 3 gave no gas. At the end of 48 hours all three solutions were removed, filtered, and precipitated with lead acetate in neutral solution in order to remove all phosphate and unchanged hexosephosphate. The excess of lead was then precipitated in the filtrates with sulphuretted hydrogen, and the filtrates, after removal of the sulphuretted hydrogen by a current of air, were neutralised and all made up to the same volume.

The following reactions for the sugars were then tried with equal volumes of each solution:—

	1.	2.	3.
a. Fehling's solution	Reduction	Slight reduction ...	No reduction
b. Mohlisch's *α*-naphthol reaction	Intense violet coloration	Violet coloration ...	No reaction
c. Seliwanoff's resorcinol reaction for fructose	Fair reaction...............	Very faint reaction	No reaction

50 c.c. of each solution were mixed with equal quantities of phenylhydrazine dissolved in acetic acid, and heated in boiling water for 1 hour. Osazones were formed in Solutions 1 and 2, but not in 3. These osazones were filtered off, washed with water, dried, washed with ether, again dried and weighed. That from No. 1 weighed 0·09 gramme; that from No. 2, 0·01 gramme. No. 1 melted at 166°—168° and after recrystallisation from boiling toluene, at 185°—186°. The osazone from No. 2 melted at 196°—198°.

Hence a sugar has been produced by the action of zymin freed from coferment on the hexosephosphate, which has the characteristic reactions of fructose, although the presence of other hexoses is not excluded.

A sugar of the same properties has already been shown to be formed when a hexosephosphate is hydrolysed by acids.*

The decomposition products of hexosephosphate by enzymic and acid hydrolysis appear, therefore, to be the same.

(*c*) Fermentation by yeast of the sugar produced by the enzymic hydrolysis of hexosephosphate.

Experiment 9.—20 grammes of zymin (Schroder) were washed as before and made to 100 c.c. with water. The following solutions were then incubated at 25° in presence of toluene:—

1. 50 c.c. of this suspension +40 c.c. of a solution of sodium hexosephosphate, equal to 27 c.c. of normal solution, and equivalent, when completely fermented, to 302 c.c. of carbon dioxide at N.T.P.
2. 50 c.c. zymin suspension +40 c.c. water.
3. 50 c.c. water +40 c.c. sodium hexosephosphate solution.

No. 1 gave a total evolution of 167 c.c. of carbon dioxide, whilst Nos. 2 and 3 gave no gas. At the end of three days the solutions were filtered, and treated as in Experiment 8. The final solutions, free from hexosephosphate, were then incubated with a suspension of well-washed living yeast, which had previously been autofermented for some days.

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* Young, 'Roy. Soc. Proc.,' B, 1909, vol. 81, p. 528.

	Gas evolved.	Gas evolved, corrected for auto-fermentation.
	c.c.	
a. 15 c.c. yeast suspension +46 c.c. No. 1	84·1	77·5
b. 15 ,, ,, +46 ,, No. 2	36·4	29·8
c. 15 ,, ,, +46 ,, No. 3	9·4	2·8
d. 15 ,, ,, +46 ,, water	6·6	

The carbon dioxide derived from the sugar produced by the enzymic hydrolysis of the hexosephosphate by the zymin is therefore equal to $77·5-(29·8+2·8) = 44·9$ c.c.

Hence the sugar formed by digesting hexosephosphate with zymin is readily fermentable by yeast.

(*d*) Production of carbon dioxide from hexosephosphate by yeast-juice and zymin.

It follows from the facts recounted above, which are expressed in equation (2) at the commencement of the paper, that the products of the enzymic hydrolysis of the hexosephosphates should be fermented by yeast-juice. Since, moreover, the enzyme producing this hydrolysis, which may be termed *hexosephosphatase*, is present in yeast-juice, the hexosephosphate itself when added to yeast-juice should undergo gradual hydrolysis and fermentation. The whole of the phosphorus would then be liberated as free phosphate, and the carbohydrate residue would be converted into alcohol and carbon dioxide.

The following experiments show that hexosephosphate is actually fermented by yeast-juice with evolution of carbon dioxide and production of free phosphate:—

Experiment 10.—Two quantities of 25 c.c. of yeast-juice were incubated at 25° in presence of toluene until a steady rate of auto-fermentation had been attained. (*a*) To one were added 15 c.c. of water; (*b*) To the other were added 15 c.c. of a solution of sodium hexosephosphate containing phosphorus equivalent to 0·4673 gramme of $Mg_2P_2O_7$ and therefore capable of yielding 94·3 c.c. of carbon dioxide.

	CO_2 evolved in 40 hours.
	c.c.
a.	85
b.	131·8
Difference......	46·8 at 19° and 762 mm. = 42·97 c.c. at N.T.P.

Somewhat less than half the available carbon dioxide was therefore evolved.

In this case the auto-fermentation of the yeast-juice was a considerable fraction of the total fermentation produced in presence of the hexosephosphate. This introduces an uncertainty, as the additional phosphate constantly being formed in (*b*) might accelerate the auto-fermentation to such an extent as greatly to increase the total evolved. In order to avoid this complication as far as possible, a second experiment was carried out with yeast-juice which was prepared from yeast previously kept in the pressed condition at 20° for 20 hours, the juice being then treated with living yeast for three hours before use in order to ferment away any free sugar. In this way a juice of small auto-fermenting power was obtained. Unfortunately the fermenting power of the juice towards sugar was also considerably lowered by this treatment.

Experiment 11.—Yeast-juice, treated as described above, was employed, and two quantities were taken—

a. 30 c.c. of the juice + 30 c.c. of a solution of potassium hexosephosphate, equivalent to 134·6 c.c. of carbon dioxide.

b. 30 c.c. of the juice + 30 c.c. of water.

These mixtures were incubated in presence of toluene, and the gas evolved and phosphate formed were estimated.

	Carbon dioxide.		Phosphate rendered free, as $Mg_2P_2O_7$.
	c.c.	gramme.	
a	52·3	0·0944	0·2058
b	14·5	0·0262	0·0792
Difference	37·8	0·0682	0·1266

Here the fermentation of the hexosephosphate has yielded at least 0·0682 gramme of carbon dioxide and 0·1266 gramme of phosphate as $Mg_2P_2O_7$.

The fact that hexosephosphate is fermented by zymin has been observed by Iwanoff,* and this observation has been confirmed by the authors.

IV. *Nature of the Chemical Change involved in Alcoholic Fermentation.*

The facts (1) that alcoholic fermentation occurs according to the equation:

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2CO_2 + 2C_2H_6O + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2,$$

and (2) that the hexosephosphates produced from glucose, fructose, and mannose appear to be identical† have an important bearing on the chemical interpretation of the decomposition by fermentation of the hexoses into carbon dioxide and alcohol. It has been pointed out by Young (*loc. cit.*) that the identity of the hexosephosphates from these three sugars may possibly be explained in either of two ways. In the first place it is to be noted that these three sugars have a common enolic form, and the hexosephosphate may be a derivative of this. In the second place, it is possible that the two molecules of sugar which are involved in the reaction, of which the equation is given above, may be decomposed into smaller groups, and that the hexosephosphate may be formed by a synthesis from these. As the formation of the hexosephosphate is invariably accompanied by that of an equivalent amount of carbon dioxide and alcohol, the second explanation appears the more probable, as it provides a source for the simultaneous production of these substances.

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* 'Centr. Bakt.,' Abt. II, 1909, vol. 24, p. 1.

† Young, 'Roy. Soc. Proc.,' B, 1909, vol. 81, p. 528.

According to this view two molecules of the hexose, or possibly of the enolic form, are each decomposed primarily into two groups. Of the four groups thus produced, two go to form alcohol and carbon dioxide, and the other two are synthesised to a new chain of six carbon atoms, which forms the carbohydrate residue of the hexosephosphate. The introduction of the phosphoric acid groups may possibly occur before the rupture of the original molecules, and may even be the determining factor of this rupture, or, again, this introduction may take place during or after the formation of the new carbon chain. Sufficient information is not yet available for the exact formulation of a scheme for this reaction. Such a scheme, it may be noted, would not necessarily be inconsistent with the views of Wohl and Buchner as to the way in which the carbon chain of a hexose is broken in the process of fermentation, but would interpret differently the subsequent changes which are undergone by the simpler groups which are the result of this rupture.

It may, however, be pointed out that if the hexosephosphate be a derivative of the enolic form common to mannose, glucose, and fructose, the compound derived from galactose should be different. If, on the other hand, the hexosephosphate be derived from fractions of two hexose groups, the galactose compound might, or might not, be different from that derived from the other hexoses. The enolic form of galactose differs from that of mannose, glucose, and fructose, in the symmetry of its γ-carbon atom, and the production of a hexosephosphate different from, or identical with, that derived from glucose, would depend on the preservation or destruction of the symmetry of this carbon atom in the reaction. It appears, therefore, that a study of the behaviour of galactose towards phosphate in the presence of the yeast enzymes may have an important bearing on the question, and experiments on this subject are now in progress.

Summary.

1. When glucose or fructose is added to yeast-juice in presence of excess of phosphate, a period of accelerated fermentation occurs, during which the added sugar undergoes the reaction :

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2CO_2 + 2C_2H_6O + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2,$$

one molecule of carbon dioxide being evolved for each molecule of sugar added.

2. When the available phosphate of a mixture of ferment, coferment, and sugar is greatly reduced, the total fermentation produced becomes very small. The addition of a small amount of a phosphate to such a mixture produces a relatively large increase in the total fermentation, even after allowing for the amount of carbon dioxide equivalent to the phosphate added.

3. A hexosephosphate when digested with yeast-juice is hydrolysed by an enzyme, hexosephosphatase, with production of free phosphate and a sugar which is capable of being fermented by yeast.

As the result of this hydrolytic action, the hexosephosphates, when treated with yeast-juice or zymin, are finally converted into carbon dioxide, alcohol, and free phosphate.

4. In discussing the chemical changes which the molecule of sugar may undergo in the process of fermentation, it is necessary to take into consideration the fact that two molecules of sugar are involved in the reaction.

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The function of phosphates in alcoholic fermentation.

[Biochemical laboratory, Lister Institute of Preventive Medicine.]

By **Arthur Harden** and **W. J. Young.**

In a series of papers (Proc. Roy. Soc. B. 1906, 77. 405; 1906. 78. 369; 1908. 80. 299; 1909. 81. 336) the authors have advanced the theory that the alcoholic fermentation of the simple sugars by yeast-juice proceeds according to the equation;

$$1)\ 2\ C_6H_{12}O_6 + 2\ R_2HPO_4 = 2\ CO_2 + 2\ C_2H_6O + 2\ H_2O + C_6H_{10}O_4\ (PO_4R_2)_2.$$

The product of the formula $C_6H_{10}O_4(PO_4R_2)_2$, which is regarded as the salt of a hexosediphosphoric acid (See Young, Proc. Roy. Soc. B. 1909. 81. 528) is thus a by-product of alcoholic fermentation. It is however hydrolysed by an enzyme present in the juice, which may be termed hexosephosphatase, into a fermentable sugar and a phosphate, according to equation (2);

$$2)\ C_6H_{10}O_4\ (PO_4R_2)_2 + 2\ H_2O = C_6H_{12}O_6 + 2\ R_2HPO_4$$

These substances then again enter into reaction (1) so that finally the whole of the sugar is fermented and the phosphate left in the free state.

The rate at which this second reaction occurs determines the steady rate of fermentation observed when glucose or fructose is fermented by yeast-juice in the absence of added phosphate, whilst the maximum rate corresponding to the first equation is only realised when phosphate is added, the amount supplied by reaction (2) being less than the maximum which can be brought into reaction by the fermenting agent of the juice.

The evidence in favour of this theory is given in full in the papers quoted above, but may be summarised briefly here:

When a soluble phosphate is added to a fermenting mixture of a hexose and yeast-juice, the following phenomena are to be observed: a) The rate of fermentation is at once greatly increased. b) This acceleration lasts for a short time and the rate then falls off and returns approximately to its original value. c) During this period the extra amount of carbon dioxide evolved and alcohol produced, in excess of the amounts which would be formed in the absence of added phosphate, are chemically equivalent to the phosphate added, according to equation (1). d) The phosphate is converted into a form which is not precipitable by ammoniacal magnesium citrate mixture, and is then present as a salt of a hexosediphosphoric acid, $C_6H_{10}O_4\ (PO_4H_2)_2$.

The hexosephosphate thus formed is gradually hydrolysed by an enzyme present in the yeast-juice. As long as excess of sugar is present and fermentation is proceeding actively, reaction (1) continues as fast as phosphate is supplied and there is no accumulation of free phosphate. After the cessation of alcoholic fermentation, however, hydrolysis continues and free phosphate then accumulates.

A similar hydrolysis of the hexosephosphate and accumulation of free phosphate occur when the residue, freed from coenzyme by filtration through compound, and there is no explanation of the observed fact that the rate rapidly falls as soon as the free phosphate has disappeared, in spite of the fact that at that moment the concentration of the hexosephosphate is at its highest.

Iwanoff's synthease. Iwanoff states in the introduction to his latest paper that he has previously shown that the conversion of phosphates into phospho-organic compounds by zymin or hefanol, does not require to be accompanied by alcoholic fermentation, but that it also occurs on the addition of phosphates to be filtrate which is obtained from a fermented mixture of sugar solution and zymin or hefanol. He therefore ascribes the formation of the hexosephosphate to a special enzyme which he terms synthease.

An examination of the experimental evidence adduced in support of this statement, however shows that although Iwanoff proved that the filtrate from a mixture of zymin, cane-sugar and water was capable of converting phosphate into hexosephosphate, he did not show that this same filtrate was incapable of producing alcoholic fermentation.

In order to ascertain the properties of a filtrate prepared in this way the following experiment, based on that of Iwanoff was made.

Exper. 3. 15 grams zymin (from top yeast) + 7.5 g. cane-sugar + 75 cc. water + toluene were allowed to stand at 15° for 24 hours. The liquid was centrifuged and filtered and to the clear filtrate 20 cc. of 9 per cent. sodium phosphate solution were added. The whole was then saturated with carbon dioxide and maintained at air temperature in presence of toluene. A vigorous fermentation set in, 56.7 cc. of gas being evolved in 20 hours.

Exper. 4. A second experiment was made with zymin (Schroder), the filtrate being obtained in the same way. 30 cc. of this filtrate at air temperature gave 112.2 cc. of carbon dioxide in 20 hours in the presence of toluene.

It appears therefore that a filtrate prepared from zymin in the manner described by Iwanoff is invariably capable of producing alcoholic fermentation.

On the other hand, when a mixture of zymin, cane-sugar and water was kept for 48 hours at 15°, the filtrate was found neither to possess the power of converting phosphate into hexosephosphate, nor that of producing alcoholic fermentation.

Exper. 5. 40 cc. of the filtrate prepared as in Exper. 4 but from a mixture kept 48 hours before filtration, + 40 cc. of 10 per cent sodium phosphate solution gave only 9 cc. of carbon dioxide in 24 hours. It was therefore practically devoid of fermenting power. The liquid was then examined for free phosphate, with the following result:

40 cc. of original filtrate	0.1048 g $Mg_2P_2O_7$
Phosphate added	1.2736 g ,,
Total Present	1.3784 g ,,
40 cc. of filtrate after 24 hours	1.3832 g ,,

Instead of a loss of free phosphate by conversion into hexosephosphate, therefore, there has been a small gain.

The results show that at present there is no evidence whatever that formation of hexosephosphate can proceed without the accompaniment of alcoholic fermentation.

Iwanoff supports his view that a special enzyme is concerned in the formation of hexosephosphoric acid (his triosophosphoric acid) by the results of experiments in which zymin and hefanol were extracted with water. When either of these materials was washed several times with water, a residue was obtained which was either altogether incapable of fermenting glucose or only produced a very small amount of fermentation either in presence or absence of added phosphate, whilst it was capable of producing a much larger evolution of carbon dioxide from a solution of the hexosephosphate. (Experiments Nos. 16—24, p. 8). From these results Iwanoff argues that extraction with water removes the synthease, but leaves behind the alcoholase, so that the residue remains capable of fermenting hexosephosphate, but not of producing it, and hence cannot ferment glucose.

The filtrate from the zymin was found by Iwanoff not to possess any power of producing fermentation, whilst that from the hefanol fermented glucose freely. (Exper. 25, p. 10).

The authors have previously shown that yeast-juice contains a dialysable, thermostable substance, which they have provisionally termed the co-ferment or co-enzyme, the presence of which along with that of the non-dialysable, thermolabile enzyme is essential for fermentation.

The method adopted by Iwanoff to remove his synthease was well adapted to remove the soluble co-enzyme and experiments were therefore made to ascertain whether this were the cause of the phenomena observed, with the following results.

1. The loss of the power to ferment glucose or fructose, which occurs when zymin is washed with water, is not due to the removal of an enzyme, since this power is restored when the boiled washings are added to the residue.

Exper. 6. 20 grams of zymin (Schroder) were centrifuged four times with 100 cc. of water at a time, and were then made up to 100 cc.

a) 15 cc. of this suspension + 2 g. Fructose + 15 cc. water + tol. gave at 25° a total of 1.2 cc. of carbon dioxide.

b) a second 15 cc. + 15 cc. of the first washings boiled, gave under the same conditions 339.7 cc.

2. When zymin is thoroughly washed with water it ferments neither glucose, fructose nor hexosephosphate, but the power of fermenting all of these is restored to it by the addition of the boiled washings.

Exper. 7. The remaining washed zymin from the foregoing experiment was filtered at the pump, twice stirred up with 50 cc. of water at a time and filtered at the pump and finally made to 50 cc.

a) 15 cc. of this suspension + 30 cc. of a solution of pure sodium hexosephosphate, containing 0.254 g. of the acid, + toluene at 25° gave only 1.4 cc. of gas.

b) A second 15 cc. + 2 g. fructose + 20 cc. of the boiled first washings gave under the same conditions 96 cc.

Exper. 8. The progressive removal of the co-enzyme by washing with water is well seen in the following experiment, although the treatment was not continued long enough to render the residue absolutely inactive to hexosephosphate.

21 g. of zymin (Schroder) were shaken for 10 minutes and then centrifuged for 15 minutes with successive quantities of 150, 120, 120 and 120 cc. of water. The residue was made to 140 cc. and two quantities of

20 cc. were taken, equivalent to 3 g. of original zymin, for experiment II. The remainder was again centrifuged and treated as before with further quantities of 120 and 120 cc. of water, made to 100 cc. and two further quantities of 20 cc. each taken for experiment III. The following experiments were then made, the washed zymin in each case corresponding with 3 grams of original zymin.

I. Fermenting power of the original zymin to hexosephosphate.

1. 3 g. unwashed zymin + 10 cc. sodium hexosephosphate solution containing 0,825 g. acid, + tol at 25° gave 104.5 cc. The total available carbon dioxide from this amount of acid is 117 cc. at air temperature.

II. Fermenting power of partially washed zymin to fructose and hexosephosphate.

2. 20 cc. of zymin suspension (after four washings) + 2 g. fructose + 8.8 cc. of water + toluene at 25° gave 1 cc. of CO_2.

3. 20 cc. of the same suspension + 10 cc. of the hexosephosphate solution + toluene at 25° gave 51.5 cc. of CO_2.

III. Fermenting power of more completely washed zymin to hexosephosphate.

4. 20 cc. of zymin suspension (after seven washings) + 10 cc. of hexosephosphate solution + 20 cc. water + toluene at 25° gave 6 cc.

5. 20 cc. of the same suspension + 10 cc. of hexosephosphate solution + 20 cc. of boiled first washings + toluene at 25° gave 88.5 cc.

It follows from these experiments that the loss of fermenting power occasioned by washing zymin with water is completely explained by the removal of the soluble thermostable co-enzyme and that there is no experimental evidence for the existence of Iwanoff's synthease. The facts that the zymin can thus be rendered quite inactive towards hexosephosphate and that the fermenting power is restored by the addition of the boiled washings also dispose of the suggestion made by Iwanoff and more recently by v. Lebedew (loc-cit) that the hexosephosphate is itself the co-enzyme[1]).

The new fact which has been brought to light by Iwanoff's experiments, that the products of the enzymic hydrolysis of hexosephosphoric acid can be fermented in the presence of smaller concentrations of co-enzyme than are necessary for the decomposition of glucose, or fructose is of considerable interest and it is hoped that the possibility of rapidly freeing zymin from co-enzyme by repeated washing with water will be of great assistance in studying this question as well as the problem of the quantitative relations between enzyme, co-enzyme, phosphoric acid and fermentable substance.

Nov. 29 th. 1909.

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[1]) The opinion that the hexosephosphate may turn out to be the co-enzyme has been erroneously attributed to the authors by several investigators. The passage upon which this misapprehension rests (Proc. Roy. Soc. B. 1908. 80. 303.) simply suggests that the co-enzyme may contain a phosphoric acid group. The authors are in possession of conclusive experimental evidence, which will shortly be published, that the hexosephosphate does not restore activity to a residue rendered inactive by gelatin-filtration of yeast-juice.

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ON THE NATURE OF THE FAT CONTAINED IN THE LIVER, KIDNEY AND HEART. Part II.

By PERCIVAL HARTLEY, *Grocers' Company Research Scholar.*

(*From the Lister Institute of Preventive Medicine.*)

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INTRODUCTION. In a paper ⁽¹⁾ published in 1907 it was shown that the fats occurring in the liver, kidney and heart differ in their properties from those found in the adipose connective tissue. The fats from the organs yield on saponification a mixture of higher fatty acids which is far more complex than the mixture obtained by saponification of the connective tissue fat. This latter consists of stearic, palmitic and oleic acid, but the fatty acids from the organs contain in addition to these three, higher fatty acids which are more unsaturated than oleic acid. Thus the fatty acids from the adipose tissue have a low iodine value (35—65) and are unaffected by heating and by exposure to air. The fatty acids from the organs on the other hand have a very high iodine value (115—135), and when exposed to the air or when heated undergo changes whereby the iodine value diminishes, and at the same time a portion of the fatty acids is converted into a resinous substance which is insoluble in petroleum ether. It was shown that the higher fatty acids from the organs are similar in their properties to the so-called drying oils, substances which contain the glycerides of acids more unsaturated than oleic acid.

Since the publication of the above paper the nature of the fats occurring in the liver has been further investigated, with the object of obtaining more exact information regarding the individual fatty acids which compose the mixture obtained by the method of preparation already described. The earlier experiments had shown that the fatty acids obtained from the liver, kidney and heart muscle are very similar in character. The most convenient source of the fatty acids, however, is the liver, and the liver of the pig is especially suitable. The large quantities of material used in the experiments, the results of which are described in the following pages, were obtained exclusively from the pig's liver.

THE FATTY ACIDS OCCURRING IN THE LECITHIN-LIKE SUBSTANCES OF THE LIVER.

It is well known that the fats occurring in the active organs of the body are present not exclusively as simple glycerides as in the case of the fat of the adipose connective tissue, but to a considerable extent as more complex substances of the nature of lecithin, jecorin, cuorin, &c. Henriques and Hansen (2) showed that the lecithin of the yolk of eggs yielded fatty acids having a high iodine value and argued from this that a considerable portion of these acids belonged to the linoleic series. More recently Erlandsen (3) has studied the lecithin-like substances occurring in the heart muscle of the ox, and he has shown that the fatty acids present in some of these substances are more unsaturated than oleic acid. It has been shown that highly unsaturated acids occur in the liver, kidney and heart, but by the methods adopted for their extraction no information was obtained concerning the manner in which these acids are combined. In the light of Erlandsen's results it appeared to be of interest to discover whether the more unsaturated acids of the liver are present as simple glycerides or in the form of more complex substances of the nature of lecithin. The following experiments were carried out in order to investigate this point.

200 grams of fresh liver tissue, reduced to a fine pulp, were shaken with 600 c.c. alcohol and allowed to stand for three hours. At the end of this time the alcoholic extract was filtered through linen, and to the residue a second quantity (600 c.c.) of alcohol added and the whole allowed to stand over-night. The alcoholic extract was separated as before, and the residue was ground up with sand and extracted a third time with alcohol. The three alcoholic extracts were added together and the alcohol distilled off at reduced pressure. The residue remaining in the flask was dissolved in a small quantity of ether, and acetone added until the addition of this fluid produced no further precipitate. The precipitate was filtered off and dissolved again in a small quantity of dry ether, and acetone added to the ethereal solution until no further precipitate was produced. The precipitate so obtained weighed 4·25 grams: it was almost pure white in colour. The iodine value was determined at once and was found to be 96·7. A portion was then saponified with alcoholic potash, and the fatty acids precipitated with sulphuric acid and extracted with petroleum ether. The iodine value of the higher fatty acids thus obtained was 143·9.

From the acetone-ether solution 2·98 grams of substance were obtained the iodine value of which was 101·9. On saponification this yielded fatty acids having an iodine value of 127·7.

The residue remaining after extraction with alcohol (sand and tissue) was extracted with ether. A small quantity of substance was obtained, iodine value 101·6.

A portion of the same liver was saponified at once in the usual way: the iodine value of the fatty acids was 129·0.

The method described above for the preparation of lecithin is one devised by Roaf and Edie(4). The solid precipitated from ethereal solution by acetone consists in the main of lecithin and similar substances; and the experiment shows that the fatty acids present in the lecithin-like substances of the liver are unsaturated acids; the iodine value being 143·9 it is clear that some of these acids are more unsaturated than oleic acid, and as some of the fatty acids obtained from this lecithin fraction were solid, saturated acids, it is probable that some of the unsaturated acids contain more than two unsaturated linkages.

The acetone-ether solution contains chiefly neutral fat, although the rather great difference between the iodine value of the original substance (101·9) and the higher fatty acids obtained by saponification (127·7) indicates that other substances are also present. The product obtained by extracting the residue with ether also consists of neutral fat, and the fairly high iodine value (101·6) shows that at least some of the unsaturated acids are present in the form of neutral glycerides[1].

SEPARATION OF THE SATURATED AND UNSATURATED ACIDS.

The separation is based upon the solubility of the lead salts of the unsaturated acids in ether, and was carried out in the following manner.

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[1] Compare Kennaway and Leathes, *Lancet*, Jan. 9th, 1909.

50 grams of the higher fatty acids were dissolved in 500 c.c. of a 2 % solution of caustic potash and the excess of alkali neutralised with acetic acid, phenol phthalein being used as indicator. 750 c.c. of a 7 % solution of lead acetate was heated to boiling and the hot solution of the soaps slowly run in. A dense precipitate was thrown down which adhered almost completely to the sides of the vessel. When cool the clear supernatant fluid was poured off and the lead soaps washed three times with hot water, and then dried. The dried lead soaps were warmed with 1·5 litres of ether; the ethereal solution was filtered off and the residue extracted again with 500 c.c. ether. The ethereal solution of lead salts was shaken with 20 % hydrochloric acid, and the lower layer of lead chloride and water drawn off. The ethereal solution of unsaturated acids was washed with water until the washings were free from acid. The ether was distilled off on the water bath, the last traces being removed by heating at reduced pressure in a stream of dry carbon dioxide. The iodine value of the unsaturated acids was determined at once.

The amount of unsaturated acids obtained in different experiments varied considerably; usually from 40 % to 50 % of the original acids were, according to this method, unsaturated. The iodine value of the unsaturated acids also varied a little and was usually from 165 to 175. The separation of saturated and unsaturated acids by the method described above, although perhaps the best available, is not a quantitative one. This is shown by the fact that the lead soaps insoluble in ether yield higher fatty acids the iodine value of which is sometimes considerable. In one case this was found to be as high as 53·2, but in others it was much lower (from 20 to 30). Other experiments were carried out in which smaller quantities of higher fatty acids were used, but no better results were obtained, some unsaturated acid always remaining unextracted.

The unsaturated acids prepared in this way are likely to be mixed with cholesterol and unsaponified matter. These were removed by extraction of the solution of the potassium salts of the acids with ether. The mean molecular weight of the purified acids was determined.

0·6281 gm. acids neutralised 20·1 c.c. $\frac{N}{10}$ NaOH. M.W. = 312·5.

0·5953 gm. acids neutralised 19·3 c.c. $\frac{N}{10}$ NaOH. M.W. = 308·4.

Since the molecular weight of stearic is 284, the results given above indicate the presence in the liver of an unsaturated acid the molecular weight of which is higher than that of stearic acid.

Distillation of the methyl esters of the unsaturated acids.

An attempt was made to separate the unsaturated fatty acids by fractional distillation. As is well known, the methyl esters of the higher fatty acids distil at a lower temperature than the corresponding fatty acids. The methyl esters of the unsaturated acids were therefore prepared and submitted to fractional distillation.

To 125 grams of unsaturated acids (iodine value 170·1) were added 200 c.c. methyl alcohol, and to the mixture 10 c.c. of concentrated sulphuric acid were slowly run in. The mixture was heated for two hours and the esters separated and collected in the usual way. On standing in an ice-room for twelve hours a crystalline solid separated out. This was filtered off, crystallised from 70 % alcohol, and was identified as cholesterol. Cholesterol appears to be less soluble in the methyl esters of the liver fatty acids than in the acids themselves; in the whole course of these experiments in which many preparations of the unsaturated acids from the liver have been made, the separation of cholesterol from the fatty acids with which it is mixed has never been observed. The iodine value of the esters was 167·4. 96·8 grams of the esters of the mixed unsaturated acids were distilled at a pressure of 3 mm.; the course of the experiment was followed by determining the iodine value of the different fractions collected. The results are shown in the following table.

TABLE I.

No. of fraction	Temp. of vapour	Weight of fraction	Iodine value
1	160°—170°	63·1 gms.	146·2
2	170°—180°	4·7 ,,	212·2
3	180°—190°	8·8 ,,	249·2
4	over 190°	4·5 ,,	249·2

The total amount of esters distilled was 81·1 grams, about 84 % of the total used in the experiment. More than two-thirds of this was collected in the first fraction. A comparison of the iodine values of the various fractions shows that this increases with increasing boiling point: a partial separation has been effected, the more unsaturated acids distilling at the higher temperatures. The first three fractions distilled as almost colourless oils, but the last fraction was yellow in colour. In an earlier paper reference was made to the ease with which the unsaturated acids of the liver undergo oxidation, and this was again illustrated in the above experiment. Towards the end of the distillation there were unmistakable signs of decomposition, and, judging from the figures obtained for the iodine values of the various fractions, it is the more unsaturated acids which decompose on heating, even at low pressures. A residue remained in the distilling flask which was dark in colour and viscid. The iodine value was 126·4.

The large fraction (1) was again distilled and a further separation was effected. 62·6 gms. esters were used and the distillation carried out at 2 mm. pressure.

TABLE II.

No. of fraction	Temperature	Weight of fraction	Iodine value
1*a*	150°—155°	29·4 gms.	127·5
1*b*	155°—159°	27·3 ,,	159·0
1*c*	over 159°	3·6 ,,	227·2

Fractions 1*c* (Table II) and 2 (Table I) were mixed together, and fractions 3 and 4 (Table I) were mixed together and redistilled. Thus four fractions were obtained for further examination, viz. 1*a*, 1*b*, (1*c* + 2) and (3 + 4).

Saponification of the esters and examination of the acids.

A weighed quantity of the esters was saponified with alcoholic potash. The resulting soap was dissolved in water and the soap solution thoroughly extracted with petroleum ether in order to remove cholesterol and unsaponified matter. The soap solutions were then acidified with 10 % sulphuric acid, and the liberated fatty acids separated in the usual way. The weight of fatty acids obtained, the iodine value and mean molecular weight were determined in each case. The results are shown in the following table.

TABLE III.

No. of fraction	Weight of esters saponified	Iodine value of esters	Weight of acids obtained	Iodine value of acids	Mean molecular weight of acids
1*a*	6·0 gms.	127·5	5·66 gms.	129·5	287
1*b*	6·0 ,,	159·0	5·47 ,,	162·3	290
(1*c*+2)	7·5 ,,	227·2 212·0	7·15 ,,	226·1	305
3+4	10·1 ,,	249·2	9·53 ,,	264·9	313

Molecular weight determinations: data.

Fraction	1*a*	0·6528 gm.	acids	neutralised	23·75 c.c.	$\frac{N}{10}$ NaOH.
,,	1*b*	0·6593	,,	,,	22·7 c.c.	,,
,,	(1*c*+2)	0·6193	,,	,,	20·3 c.c.	,,
,,	(3+4)	0·6191	,,	,,	19·8 c.c.	,,

The bromine absorption of the different fractions.

The higher fatty acids which contain three or more unsaturated linkages within the chain form addition products with bromine which are insoluble in the common organic solvents, while oleic and linoleic acids form addition products which are easily soluble. The "hexabromide test" is based upon these facts, and it is used for determining the amount of linolenic acid, or acids more unsaturated than this, in a mixture of unsaturated acids. The amount of insoluble addition products formed when the fatty acids from each of the four fractions mentioned above are treated with bromine was determined in the following way.

In each case a known weight of acid was used. The amount of bromine required to unite with the unsaturated acids was calculated and a slight excess of this quantity was used. The acids were dissolved in glacial acetic acid and the solution cooled to 5° C. The bromine was also dissolved in glacial acetic acid and was added very slowly to the solution of the acids. Rise of temperature was avoided and in no case was the evolution of hydrobromic acid observed. After the addition of the bromine the whole was allowed to stand for three hours and the separated solid was then filtered off, washed first with cold acetic acid, then with cold alcohol and finally with cold dry ether. The precipitate, which was pure white in colour in each case, was dried and weighed. The results are collected in the following table.

TABLE IV.

No. of fraction	Weight of acids used	Bromine added	Weight of insoluble bromination product	Total (sol.+insol.) bromination product	Insol. bromination product, %
1*a*	3·67 gms.	1 c.c.	0·246 gms.	6·66 gms.	3·7
1*b*	3·65 ,,	1 c.c.	0·702 ,,	7·38 ,,	9·5
(1*c*+2)	3·00 ,,	2 c.c.	2·260 ,,	7·27 ,,	31·1
(3+4)	3·00 ,,	2 c.c.	3·311 ,,	8·00 ,,	41·4

The following conclusions may be drawn from the results given in the four preceding tables.

1. Fractional distillation of the methyl esters of the unsaturated acids from the liver effects a partial separation.

2. The fractions 1*a* and 1*b* consist chiefly—the former almost entirely—of oleic and linoleic acids; fractions (1*c* + 2) and (3 + 4) contain large quantities of acids more unsaturated than linoleic acids.

3. The molecular weights of the acids of the first two fractions are of the same order as that of stearic acid, and indicate that the unsaturated acids of these two fractions contain eighteen carbon atoms. The molecular weights of the acids of the last two fractions however are much higher and point to the presence of acids containing twenty carbon atoms.

4. The fact that the fractions yielding acids of high molecular weight have also high iodine values suggests that the two properties may be related; that is, the acids of high molecular weight may be the acids more unsaturated than linoleic acid.

THE ACTION OF BROMINE ON THE UNSATURATED ACIDS.

These experiments were carried out with the object of securing somewhat larger quantities of the insoluble bromination products, in order to study their properties and subject them to analysis. Further, a determination of the amount of insoluble bromination products provides a means of estimation of acids of the type $C_nH_{2n-6}O_2$ and $C_nH_{2n-8}O_2$ present in the mixture of fatty acids from the liver.

A weighed quantity of the mixed (saturated and unsaturated) fatty acids from the liver was treated with bromine and the insoluble products of bromination were separated in the manner already described. Now linoleic hexabromide is soluble in benzene, whereas the octobromides are quite insoluble. The insoluble precipitate was therefore extracted with benzene in order to remove any hexabromide present in the mixture. The extraction with benzene was extended over many days and was continued until no more solid was extracted. In this way a small amount of solid was extracted which melted with decomposition at 222°—224°, but analysis showed that the substance was certainly not a hexabromide. By far the greater quantity was insoluble in benzene, and analyses of different preparations gave the following figures:

(1) {0·1577 gm. substance gave 0·2498 gm. AgBr. Br = 67·4 %.
0·1489 gm. substance gave 0·2359 gm. AgBr. Br = 67·4 %.

(2) 0·1571 gm. substance gave 0·2492 gm. AgBr. Br = 67·5 %.

In a third preparation extraction with benzene was followed by extraction with ethyl acetate, and subsequent analysis gave the following figures:

(3) 0·1927 gm. substance gave 0·3073 gm. AgBr. Br = 67·8 %.
Calculated for $C_{20}H_{32}O_2Br_8$. Br = 67·8 %.

The substance is therefore octobromoarachidic acid, and is derived from an acid in the liver, $C_{20}H_{32}O_2$, having four unsaturated linkages in the chain. An acid of this composition is stated to occur in herring oil (Bull(5)) and Tsujimoto(6) has prepared octobromostearic acid, $C_{18}H_{28}O_2Br_8$, from the fatty acids from sardine oil. Heyerdahl(7) prepared an octobromide from the fatty acids of cod-liver oil to which he ascribed the formula $C_{17}H_{26}O_2Br_8$.

The properties of the octobromoarachidic acid prepared from the liver fatty acids are in complete agreement with those of the similar substances prepared by other workers from other sources. It is a white solid which, on heating, becomes discoloured at about 220°, but it does not melt when heated in a capillary tube in the ordinary way. On platinum foil it first melts to a dark brown liquid and then burns away completely. It is quite insoluble in all organic solvents; hence it cannot be purified by the usual method, viz. crystallisation. On account of the easy solubility of the other products of bromination however, the prolonged extraction with various solvents adopted in all these experiments renders it highly probable that the octobromoarachidic acid was obtained almost, if not quite, pure, and the figures given for the analysis of the three different specimens confirm this. Other evidence of the presence of the acid $C_{20}H_{32}O_2$ has been obtained and is referred to later.

The amount of the acid $C_{20}H_{32}O_2$ present in the liver fatty acids was determined on two different samples and amounted to 7·8 % and 8·5 % in the two cases.

It may be noted here that in a long series of experiments on the action of bromine on the liver fatty acids, linolenic hexabromide has never been found among the products of bromination. Further, hexahydroxystearic acid has not been found among the products of oxidation of the liver fatty acids, in the experiments described below. Linolenic acid therefore does not appear to be present in the liver.

THE OXIDATION OF THE UNSATURATED ACIDS OF THE LIVER.

Hazura demonstrated the presence of various unsaturated acids in different vegetable oils by oxidising the mixed acids with alkaline permanganate and isolating the oxidation products. Thus, oleic acid yields dihydroxystearic acid, linoleic acid yields tetrahydroxystearic acid, and linolenic acid yields hexahydroxystearic acid on oxidation. From the fatty acids of linseed oil, for example, Hazura obtained the three hydroxy acids mentioned above, and thus demonstrated the presence of oleic, linoleic and linolenic acids in the original oil (8).

Now the liver fatty acids are characterised by the presence of many unsaturated acids, and according to Hazura's rule should yield a variety of hydroxy acids on oxidation with alkaline permanganate, and the study of such oxidation products should yield some information concerning the unsaturated acids occurring in the liver.

For the purpose of these experiments it was necessary to use large quantities of material, as the amount of hydroxy acids formed on oxidation is very small. The method adopted is illustrated in the following experiment.

50 grams of mixed (saturated and unsaturated) acids were dissolved in 50 c.c. caustic potash solution (s.g. 1·145). The resulting soap was dissolved in two litres of water and the solution cooled to 0°. Two litres of a 1½ °/₀ solution of potassium permanganate, previously cooled to 0°, were run in in a thin stream, the whole mixture being stirred with a turbine and the temperature maintained at the freezing point. When all the permanganate had been added, the solution was stirred for ten minutes, and a stream of sulphur dioxide was then passed in until the manganese dioxide was dissolved and the reaction of the fluid was acid. A white flocculent precipitate separated which was filtered off, dried on a porous plate and extracted in a Soxhlet with petroleum ether (B.P. 35°—65°). The greater portion of the precipitate was dissolved out by the petroleum ether (A). The residue which remained was extracted with hot ether until all the ether soluble substances (B) were removed. The residue (C) remaining was treated in the manner described below.

The filtrate was exactly neutralised with caustic potash solution. Some manganese dioxide separated out and this was filtered off and the clear solution was evaporated on the water bath until the volume measured about 200 c.c. Sulphuric acid (10 °/₀) was added and a strong smell like that of butyric acid was immediately observed. At the same time a fine white precipitate (D) separated which was removed by centrifuging. The clear liquid was then distilled in steam until all the volatile acids (E) had passed over. The residue (F) in the distilling flask consisted chiefly of inorganic salts, together with a considerable amount of tarry matter.

Each of the fractions obtained was then submitted to further examination.

A. *The portion soluble in petroleum ether* (*saturated acids*).

The following yields were obtained in different experiments:

(1)	50 grams mixed acids gave	28·4	grams.	56·8 °/₀.
(2)	50 ,, ,, ,,	28·7	,,	57·4 °/₀.
(3)	85 ,, ,, ,,	46·5	,,	54·7 °/₀.

This portion contains the saturated acids, the unsaturated acids which have escaped oxidation, and cholesterol. It was found that oxidation of the unsaturated acids was not complete, as on examination of the fatty acids in the three cases quoted above the iodine value was 42·6, 37·0 and 49·2 respectively. In other experiments in which more permanganate was used and the oxidation allowed to proceed for a longer time the iodine values were very much smaller, but the yield of hydroxy acids was somewhat less, owing to further oxidation having taken place.

Cholesterol was removed by extraction of the soap solutions with ether, and the mean molecular weight of the purified acids was determined.

(1) 0·6052 gm. acids neutralised 22·4 c.c. $\frac{N}{10}$ NaOH. M.W. = 270.
(2) 0·7493 ,, ,, 27·0 c.c. ,, M.W. = 277.
(3) 1·1828 ,, ,, 43·45 c.c. ,, M.W. = 272.

The molecular weight lies between that of stearic acid 284 and palmitic acid 256. But acids of lower molecular weight such as myristic acid, or of higher molecular weight such as arachidic acid, may also be present. To determine if this were the case, the methyl esters of the mixed acids were prepared and submitted to fractional distillation at reduced pressure.

45 grams of mixed esters were distilled at a pressure of 4 mm., and four fractions were collected. A portion of each fraction was saponified and the mean molecular weight of the acids determined in each case. On cooling, the esters collected in each fraction solidified to a beautifully crystalline mass. The results are collected in the following table.

TABLE V.

No.	Temp. of vapour	Weight of fraction	Mean mol. weight of acid
1	166°—170°	19·5 gms.	257
2	170°—175°	3·1 ,,	260
3	175°—180°	12·8 ,,	272
4	180°—190°	7·1 ,,	280

Molecular weight data.

(1) 1·0474 gm. acid neutralised 40·7 c.c. $\frac{N}{10}$ NaOH.
(2) 0·8147 ,, ,, 31·25 c.c. ,,
(3) 0·7995 ,, ,, 29·4 c.c. ,,
(4) 0·5500 ,, ,, 19·6 c.c. ,,

The acids from fraction 1 were recrystallised twice from alcohol and the crystals melted at 55°—56° and solidified at 54°. Analysis gave the following figures:

0·1919 gm. substance gave 0·5238 gm. CO_2 and 0·2115 gm. H_2O.
C = 74·4 %, H = 12·2 %.
Calculated for $C_{16}H_{32}O_2$. C = 75·0 %, H = 12·5 %.

The acids from fraction 3 were recrystallised twice from alcohol. Large glistening plates separated which melted at 68·6°—69·2°.

Analysis:

0·0955 gm. substance gave 0·2662 gm. CO_2 and 0·1111 gm. H_2O.
C = 76·0 %, H = 12·9 %.
Calculated for $C_{18}H_{36}O_2$. C = 76·0 %, H = 12·7 %.

Stearic and palmitic acids are therefore present in the liver.

B. *The portion soluble in diethyl ether and insoluble in petroleum ether (dihydroxystearic acid).*

This was a white crystalline substance, slightly soluble in cold ether and more soluble in the hot solvent. The following amounts were obtained in three experiments.

(1)	50 grams mixed acids gave	1·72 grams.	M.P. 120°—123°.
(2)	50 ,, ,,	2·19 ,,	M.P. 122°—124°.
(3)	85 ,, ,,	2·17 ,,	M.P. 120°—123°.

The crude substance was boiled with a large volume of water and filtered through a hot filter. The residue insoluble in water was recrystallised from 70 % alcohol. After a few crystallisations the melting point rose to 129·5°, and this did not alter after further recrystallisations from alcohol or any other solvent. On analysis the following figures were obtained:

(i) 0·1139 gm. substance gave 0·2859 gm. CO_2 and 0·1158 gm. H_2O.
$C = 68{\cdot}4\ \%$, $H = 11{\cdot}3\ \%$.

(ii) 0·1077 gm. substance gave 0·2702 gm. CO_2 and 0·1105 gm. H_2O.
$C = 68{\cdot}4\ \%$. $H = 11{\cdot}4\ \%$.

Calculated for $C_{18}H_{36}O_4$. $C = 68{\cdot}4\ \%$, $H = 11{\cdot}4\ \%$.

The substance is therefore dihydroxystearic acid and is derived from the oleic acid present in the liver.

Dihydroxystearic acid is soluble in ether, alcohol, acetone and ethyl acetate and crystallises from any of these solvents, generally in the form of hexagonal prisms. It is also slightly soluble in chloroform and in boiling water, and differs in these respects from the dihydroxystearic acid described by other observers and prepared by them from different sources, and it differs from other dihydroxystearic acids in melting point. Thus Edmed(9) gives 134° as the melting point of a dihydroxystearic acid prepared and studied by him, and Le Sueur(10) synthesised a dihydroxystearic acid which melted at 126°. The reason for these wide differences in melting point is almost certainly due to the fact that a number of isomeric oleic acids exist which on oxidation yield different dihydroxystearic acids. At present only three such acids are known. One is present in olive oil and the constitution of this acid was determined by Edmed, who showed that the double linkage is situated exactly in the middle of the chain. Le Sueur synthesised an oleic acid in which the double linkage is situated between the a and β carbon atoms. A third oleic acid is known in which the double linkage lies between the eighth and ninth carbon atoms.

The differences observed in the melting point and solubilities of the dihydroxystearic acid prepared from the liver suggest that it is derived from an oleic acid in which the double linkage is situated in a position different from that occupied by the double linkage in the oleic acid from other sources. The following experiment was carried out in order to investigate this point.

7·55 grams of dihydroxystearic acid, M.P. 129°, were dissolved in 250 c.c. of a solution containing 2·5 grams of caustic potash, and the solution heated on the water bath. A solution of potassium permanganate, containing 8 grams of the salt in 600 c.c. water, was added in small quantities at a time. An immediate reduction of the permanganate occurs, manganese dioxide being precipitated. When all the permanganate had been added the solution was cooled and the manganese dioxide filtered off. The filtrate was acidified with 10 $^0/_0$ sulphuric acid and the white flocculent precipitate (A) which separated was removed by centrifuging. The clear liquid was poured off and distilled in steam when a few drops of oil passed over. The distillation was continued until all volatile acid had passed over. Two preparations of the barium salt and one of the calcium salt of the volatile acid were made and analysed.

(1) 0·3390 gm. barium salt gave 0·2122 gm. $BaSO_4$. Ba = 36·8 $^0/_0$.
(2) 0·1799 ,, ,, ,, 0·1128 ,, Ba = 36·9 $^0/_0$.
(3) 0·1262 gm. calcium salt gave 0·0630 gm. $CaSO_4$. Ca = 14·7 $^0/_0$.
Calculated for $(C_6H_{11}O_2)_2Ba$. Ba = 37·4 $^0/_0$.
,, ,, $(C_6H_{11}O_2)_2Ca$. Ca = 14·8 $^0/_0$.

The volatile acid formed in the further oxidation of dihydroxystearic acid is therefore caproic acid. The smell of this acid was very pronounced when sulphuric acid was added to the barium and calcium salts.

The residue remaining in the distilling flask was evaporated to very small bulk and cooled. No solid separated, even on long standing. Ammonia was added until the solution was strongly alkaline, the whole boiled and calcium chloride solution added. A white precipitate was thrown down which was filtered off, dried and analysed.

0·3537 gm. calcium salt gave 0·1572 gm. CaO. Ca = 31·7 $^0/_0$.
Calculated for C_2O_4. Ca. Ca = 31·3 $^0/_0$.

Oxalic acid is therefore a product of the oxidation of dihydroxystearic acid. The analysis shows that no other soluble dibasic acid of the oxalic series is formed on oxidation.

The formation of caproic acid shows that the hydroxyl groups in the dihydroxystearic acid are attached to the sixth and seventh carbon atoms, reckoned from the methyl-group end of the chain. The other acid produced by the oxidation of dihydroxystearic acid must be decamethylene dicarboxylic acid $[CH_2]_{10}\cdot(COOH)_2$. This substance is but slightly soluble in water, and melts at 124°. It was therefore sought for in the precipitate (A). Analysis of the calcium salt showed that this consisted of a mixture of decamethylene dicarboxylic acid and unchanged dihydroxystearic acid. The melting points of these two substances lie very close together and the melting point of the mixture was 123°—125°. Attempts were made to separate the two, but owing to the small amount of material available and the similarity of the solubilities of the two acids these were not successful. An attempt to remove the dihydroxystearic acid by treatment with more permanganate led to the formation of more caproic acid and more oxalic acid, and it is possible that under the conditions of the experiment decamethylene dicarboxylic acid is destroyed, oxalic acid being formed.

There can be no doubt that the oleic acid in the liver is different from the oleic acid found in olive oil. It yields a dihydroxystearic acid on oxidation which differs, in melting point and in solubilities, from the corresponding substance described by Edmed. And the formation of caproic acid on oxidation shows that the double linkage lies between the sixth and seventh carbon atoms, reckoned from the methyl-group end of the chain.

Experiments were then carried out in order to determine whether the oleic acid occurring in the adipose connective tissue is similar to the oleic acid of the liver. The fatty acids from the adipose connective tissue of the pig were first prepared in the ordinary way, and the oleic acid separated from the mixture by the lead-salt-ether method. The iodine value of the oleic acid was 94·8. It was oxidised at 0° with potassium permanganate and the dihydroxystearic acid thus obtained, after recrystallisation from ether, melted at 134°. The further oxidation with potassium permanganate was carried out in the manner already described: the volatile acid was distilled off and converted into the acid amide by the method of Aschan (11). The amide, after crystallisation from petroleum ether and then from water, melted at 93°—94°, and on analysis gave the following figures.

0·1757 gm. amide gave ammonia corresponding to 10·8 c.c.

$$\frac{N}{10} NH_3. \quad N = 8{\cdot}6\ \%.$$

$$\text{Calculated for } C_9H_{19}NO. \quad N = 8{\cdot}9\ \%.$$

The volatile acid, formed by the oxidation of the dihydroxystearic acid derived from the oleic acid occurring in the adipose tissue, is therefore pelargonic (nonylic) acid, and the results show that the double linkage in the original oleic acid is situated exactly in the middle of the chain.

C. *The portion insoluble in cold water, petroleum ether and diethyl ether (tetrahydroxystearic acid).*

This was a pale yellow solid, and the yields obtained in different experiments varied considerably. In three cases, in each of which

50 grams of mixed acids were used, 6·2 grams, 4·4 grams and 7·9 grams were obtained. In the light of Hazura's researches this should consist of tetrahydroxystearic acid, derived from the linoleic acid present in the original acids. The purification of the crude substance was carried out as follows.

10 grams of the crude substance were boiled with three litres of water and filtered through a hot filter. On cooling the filtrate deposits small crystals: these were filtered off, dried and the melting point determined. The residue was then boiled again with three litres of water and the process repeated until the whole of the substance soluble in boiling water was extracted. In one experiment six extractions were made, the melting point of the various fractions being as follows: (i) 155°—157°, (ii) 156°—159°, (iii) 157°—161°, (iv) 164°—169°, (v) 170°—173°, (vi) 172°—173°. Fractions (v) and (vi) were mixed together, recrystallised first from 70 % alcohol and then from boiling water when a beautifully crystalline substance was obtained which melted at 175°. On analysis the following figures were obtained:

0·1116 gm. substance gave 0·2545 gm. CO_2 and 0·1005 gm. H_2O.
$C = 62{\cdot}2\ \%$, $H = 10{\cdot}0\ \%$.
Calculated for $C_{18}H_{36}O_6$. $C = 62{\cdot}1\ \%$, $H = 10{\cdot}3\ \%$.

The substance is therefore tetrahydroxystearic acid. The formation of this body on oxidation shows that linoleic acid is present in the mixed fatty acids obtained from the liver. The statement has been made that linoleic acid does not occur in animal oils. (Watts' *Dictionary of Chemistry*, Vol. III. p. 147.) But this is not the case: linoleic acid is the most plentiful of the unsaturated acids occurring in the liver.

In these experiments the amount of pure tetrahydroxystearic acid, melting at 175°, obtained was small, and a certain amount of evidence was obtained to show that another tetrahydroxystearic acid is formed by the oxidation of the mixed acids from the liver. The earlier fractions obtained by extraction of the crude substance with water are larger than, and possess a different melting point from, the later fractions from which a pure product was obtained. A large number of isomeric linoleic acids may exist according to the position of the double linkages in the chain, and these acids would yield on oxidation tetrahydroxystearic acids differing from one another in the positions occupied by the hydroxyl groups in the molecule. That such isomeric tetrahydroxystearic acids exist is rendered probable by the fact that when the unsaturated acids from different vegetable oils are oxidised, tetrahydroxystearic acids melting at different temperatures are obtained[1]. It is possible that two linoleic acids occur in the liver, one of which yields a hydroxy acid melting at 175°, and another which gives on oxidation a hydroxy acid, more soluble in water, and melting at a lower temperature. This would be present in the earlier fractions extracted, and it was found that these fractions after repeated crystallisations from different solvents gave a product the melting point of which was below 160°. The isomerism of the linoleic acids may be similar to that observed in oleic and elaidic acids; that is, the double linkages may be similarly situated in the molecule, but the arrangement of the atoms around these linkages may be different in the two cases. On the other hand the two tetrahydroxystearic acids may be derived from two linoleic acids in which the double linkages are differently situated in the two cases.

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[1] The writer has found that when the fatty acids occurring in cotton-seed oil are oxidised, two tetrahydroxystearic acids are obtained, melting at 155·5°—156·5° and 173° respectively. This fact was also observed by Hazura.

These points are being investigated, but the experiments are not yet complete. An experiment on the further oxidation of the tetrahydroxystearic acid obtained from the liver fatty acids was carried out, and the results are given below.

4·78 grams of tetrahydroxystearic acid were oxidised with alkaline permanganate in the manner already described. The manganese dioxide was filtered off and the solution acidified with sulphuric acid. A small amount of solid separated, which was centrifuged off, and the clear liquid was distilled in steam. The first 100 c.c. of distillate were collected and examined separately. A few drops of colourless oil appeared on the surface. The barium salt was prepared and analysed.

0·1793 gm. barium salt gave 0·1342 gm. $BaSO_4$. Ba = 44·0 %.

Two litres of distillate were then collected, and the barium salt of the volatile acid prepared and analysed. No insoluble oil was observed in this portion of the distillate.

0·2556 gm. barium salt gave 0·2316 gm. $BaSO_4$. Ba = 52·1 %.
Calculated for barium acetate $(C_2H_3O_2)$ Ba. Ba = 53·8 %.

The results show that when tetrahydroxystearic acid is oxidised with alkaline permanganate a mixture of volatile acids is produced. The analyses of the barium salts given above are not very conclusive. The latter portion of the distillate appears to consist of acetic acid. The presence of an insoluble oil in the first portion of the distillate together with the strong smell of caproic acid noticed when acid was added to the barium salt suggest the presence of caproic acid in this fraction, but other acids of lower molecular weight are also present. On the assumption that tetrahydroxystearic acid behaves like dihydroxystearic acid on heating with alkaline permanganate, the former substance should yield, under these circumstances, one volatile acid of the acetic series and two dibasic acids of the oxalic series. The tetrahydroxystearic acid from the liver yields acetic and caproic acids on oxidation, however, and it is possible that the acetic acid is formed from malonic acid which is first produced by the cleavage of the tetrahydroxystearic acid. Many of the acids of the oxalic series are readily converted, by loss of carbon dioxide, into monobasic acids containing one carbon atom less than the original dibasic acid.

D. The portion soluble in water (octohydroxyarachidic acid).

This fraction of the oxidation products was first crystallised from 50 % alcohol and then from hot water, in which it is easily soluble, until the melting point remained constant at 195°. It crystallises in small rectangular prisms. The amount of pure substance obtained from 200 grams of mixed fatty acids from the liver was about half a gram.

Analysis:

0·1425 gm. substance gave 0·2852 gm. CO_2 and 0·1182 gm. H_2O.
$C=54·6\ {}^0/_0$, $H=9·2\ {}^0/_0$.
Calculated for $C_{20}H_{40}O_{10}$. $C=54·5\ {}^0/_0$, $H=9·1\ {}^0/_0$.

The substance is therefore octohydroxyarachidic acid, and its formation in these experiments shows that an acid, $C_{20}H_{32}O_2$, containing four unsaturated linkages, must be present in the liver.

E. The volatile acids.

The steam distillates from four experiments, in each of which 50 grams of fatty acids were used, were neutralised with caustic soda solution and evaporated to dryness on the water bath. The residue was dissolved in the smallest possible quantity of water and 10 ${}^0/_0$ sulphuric acid added in slight excess. An oil separated and floated on the surface of the fluid. The whole was shaken out several times with ether, the ethereal extract dehydrated and the solvent distilled off from the water bath. The oil which remained was distilled from a small flask: five fractions were collected:

No.	Temp.	Weight of distillate
1	Below 100°	0·511 gm.
2	100°—125°	0·490 ,,
3	125°—200°	0·403 ,,
4	200°—210°	0·905 ,,
5	210°—215°	0·229 ,,

Each fraction was then examined in the following way:

Fraction 1. This was completely soluble in water. Ammonia was added and the solution boiled until the reaction was neutral. A drop of ferric chloride was added to the neutral solution: a red colouration was produced and on boiling a reddish brown precipitate was thrown down. Silver nitrate solution was added to another portion of the neutral solution: on warming reduction to metallic silver occurred. Mercuric chloride solution was added to a third portion of the neutral solution: on warming reduction to mercurous chloride occurred.

Formic acid is present in this fraction of the volatile acids.

Fraction 2. The barium salt was prepared and analysed.

0·3786 gm. barium salt gave 0·3056 gm. $BaSO_4$. $Ba=47·5\ {}^0/_0$.
Calculated for $(C_2H_3O_2)_2\,Ba$. $Ba=53·8\ {}^0/_0$.

This fraction contains acetic acid. On adding sulphuric acid to the barium salt, a strong smell of acetic acid was observed.

Fraction 3. A colourless oil which floated on water. Nearly the whole distilled at 190°—200°: the barium salt gave the following results on analysis.

0·3483 gm. barium salt gave 0·2340 gm. $BaSO_4$. $Ba = 39·4\ {}^0/_0$.
Calculated for $(C_6H_{11}O_2)_2Ba$. $Ba = 37·4\ {}^0/_0$.

The greater portion of this fraction consists of caproic acid.

Fraction 4. This was by far the largest fraction—a colourless oil which floated on water. Analysis of the silver salt gave the following results.

0·1921 gm. silver salt gave 0·0933 gm. Ag. $Ag = 48·5\ {}^0/_0$.
Calculated for $C_6H_{11}O_2Ag$. $Ag = 48·4\ {}^0/_0$.

This fraction consists of pure caproic acid.

Fraction 5. The analysis of the barium salt showed that this fraction also consisted of caproic acid.

0·2085 gm. barium salt gave 0·1335 gm. $BaSO_4$. $Ba = 37·6\ {}^0/_0$.
Calculated for $(C_6H_{11}O_2)_2Ba$. $Ba = 37·4\ {}^0/_0$.

The volatile acids formed by the action of alkaline permanganate on the liver fatty acids are doubtless secondary oxidation products. Caproic acid was found and identified over and over again, and arises from the further oxidation of the dihydroxystearic acid. The acetic acid and formic acid are possibly formed from acids of the oxalic series: malonic acid, for example, may be the source of the acetic acid[1]. Pelargonic (nonylic) acid was not found, and this seems to indicate that the oleic acid found in the adipose issue, which yields this substance on oxidation, is absent from the liver.

F. *The residue.*

No crystalline products were obtained from the residue remaining in the distilling flask. The large amount of inorganic salts and tarry matter present rendered the examination difficult. After removal of the tarry matter the residue reduced potassium permanganate in acid solution.

DISCUSSION OF RESULTS.

The experimental results recorded in the foregoing pages, and in a previous paper (1), show very clearly that the fats occurring in the liver, kidney and heart differ from the fats stored in the adipose connective tissue, and this difference is far greater than has formerly been supposed. One of the most striking of these differences is that while the stored fat consists of a mixture of substances, two of which at least are among the most stable of those found in the body, the organ fats are characterised by the ease with which they are oxidised. It has been shown, for example, that mere exposure to the air brings about changes in those fats which are characteristic of the organs whereby their chemical properties are strikingly modified. And it is a fact of considerable physiological importance that these easily oxidised fats are situated in those organs of the body which are the centre of greatest metabolic activity. When an animal richly stocked with fat is starved, the fat stored in the adipose tissue gradually disappears. Fat is a valuable source of energy, and in starvation the supply of stored fat is drawn upon as the needs of the organism demand. But in a fattened animal a great portion of this stored fat is stearin and palmitin, and these substances are not easily oxidised, even in the laboratory when subjected to violent treatment involving the use of high temperatures and strong oxidising agents. Yet the body provides the means of oxidising these stable substances, and moreover is capable of doing so at the ordinary body temperature. The exact manner in which this is carried out is unknown, but it is certain that the conversion of a substance like stearin to the final products of oxidation, carbon dioxide and water, does not take place in one violent reaction. The change is a gradual one, brought about in stages which follow one another in a regular, although it may be rapid manner. And a possible explanation of the occurrence of highly unsaturated, easily oxidised higher fatty acids in the liver is to be found in the assumption that such acids represent some intermediate stage through which fats pass in the course of their metabolism. The presence of these acids in the liver is conclusively proved from the results of the experiments described in this paper, and a good deal of evidence has been obtained to show that the fats of the heart muscle and kidney are similar to the fats of the liver. The iodine value is of the same order, and Erlandsen (3), working with the fats from the heart muscle, mentions the fact that these fats are easily oxidised by exposure to the air, in much the same way as has been shown to be the case with the liver fatty acids.

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[1] Compare Barrowcliff and Power. *Journal of the Chemical Society*, p. 568. 1907.

In the light of the results recorded in this paper the views of Leathes (12) concerning the breakdown of fat in the body may here be quoted.

"It seems therefore that though the fat is deposited in the connective tissues unchanged, changes subsequently take place in it, with the result that it contains more of the unsaturated acids, before it is used in the organs in which it is broken up. The unsaturated linkages become more numerous, presumably because it is at these points that the chains of carbon atoms are to break. If we could catch the process at a more advanced stage we should find that some of the unsaturated acids had disappeared, and the mean molecular weight of the acids had diminished."..."We may expect that the fatty acids undergo oxidation step by step,...that an unsaturated linkage is the first move towards this oxidation and probably the formation of a saturated oxyacid the second; the first of these preparatory changes takes place either in the organs where the oxidation is carried out or before it reaches them; but after it leaves the storage places, possibly in the liver."

That fat under certain conditions migrates from the connective tissues to the liver was shown by the experiments of Lebedeff (13), of Rosenfeld (14) and of Leick and Winckler (15). But the significance of such a migration was not appreciated. The results recorded in this and in the earlier paper (1) confirm the hypothesis that it is to undergo a change, by which the fatty acids are rendered more liable to oxidation and to be broken up into smaller molecules, that they migrate to the liver. Leathes and L. Meyer Wedell have recently shown (16) too that a portion of the fat absorbed from the intestine is conveyed to the liver, though none of it could be traced in the other organs. That which had been taken up by the liver, however, had been altered and become more unsaturated. Now the results recorded in the previous paper (1) show that the fatty acids of the fats found in the other organs are as unsaturated as those found in the liver. It seems therefore fair to infer that it is in the liver that desaturation of fatty acids is effected and that the other organs are provided with the unsaturated fatty acids which they require by the liver.

This desaturation constitutes the first steps towards the breaking up of the fatty acids into fragments that are more easily oxidised. And as appears from the results of the examination of the different varieties of fatty substances in the liver by Kennaway and Leathes this desaturation is effected in the simple glycerides in this organ, and it is not only the phosphatides that are involved. That fats and oils are broken up into smaller molecules in the manner indicated, especially if the constituent fatty acids are unsaturated, is well known. It is not only in the action of permanganate, of which instances have been given in this work, that such a demolition of long chains occurs; it occurs too in rancescence and also in the changes undergone by the "drying" oils when subjected to the process known as "blowing" (*i.e.* treatment with a stream of heated air); and in both cases the presence of unsaturated linkages is a determining factor. Hydroxy acids are formed, and with them, probably formed from them, are found lower volatile acids. Von Fürth (18) has described similar changes occurring during germination of seeds, a lowering of the iodine and acetyl values and the formation of acids of a lower molecular weight.

The occurrence of an oleic acid in the liver different from that found in the connective tissue must be interpreted as due either to the insertion of a double linkage, by the removal of a hydrogen atom from each of the 6th and 7th carbon atoms, in stearic acid, or to a change in the position of the unsaturated linkage of the oleic acid that is found in the connective tissues. On the hypothesis of the function of the liver described above it is more probable that it is the former of these changes that occurs. And the occurrence of a linoleic acid in the liver with the unsaturated linkages between the 6th and 7th and between the 9th and 10th carbon atoms, for which evidence is adduced in this paper, would be most readily understood by supposing that the oleic acid of the connective tissues had acquired a new double linkage, and that in the same position as that which is inserted by the liver into stearic acid, in converting this acid into the hepatic oleic acid. The same reaction, the formation of an unsaturated linkage between the 6th and 7th carbon atoms, in the one case in stearic acid, in the other in the oleic acid of the connective tissues, would then account for the two unsaturated acids of the liver for the constitution of which evidence has been brought forward in this paper. It may be supposed that the products of further oxidation, amongst them caproic and acetic acid, are substances the combustion of which presents no difficulty in the cells of the body. That this is the case with acetic and caproic acid is well known. The recent work of Dakin (19) on the oxidation of such lower fatty acids by hydrogen peroxide and the numerous resemblances which he has found between the course of such oxidation and that of the oxidations in the body suggest what the subsequent fate of these substances in the body is likely to be.

The occurrence of the acid with a chain of 20 carbon atoms and four unsaturated linkages, described in this paper, is not so easy to interpret. It may be that arachidic occurs in the fat of the pig, as it does in butter-fat. And if so this remarkable hydroxy acid may be the product of the desaturating activity of the liver on the saturated acid with 20 carbon atoms. The amount of it that has been found in the liver is however not small, while in lard arachidic acid is certainly not likely to occur in more than traces. So that it is probable that some other explanation may be necessary to account for its presence. This may possibly be that it is an instance of the synthetic activity of the liver in building up fatty acid chains. Evidence for such activity has been given by Magnus Levy⁽²⁰⁾ and by Leathes⁽²¹⁾. If the scheme adopted by them to account for the synthesis of fatty acids from carbohydrates corresponds to the truth, if that is this synthesis is based on the condensation of aldehyde with the formation of hydroxy aldehydes then unsaturated acids are likely to be the product of one stage of this construction; just as for instance crotonic acid is formed from β oxybutyric aldehyde (aldol), and as caprylic acid was obtained by Raper⁽²²⁾ from an unsaturated hydroxy aldehyde with eight carbon atoms. The formation of unsaturated fatty acids from carbohydrates and their occurrence in the liver I have myself observed. A number of guinea-pigs were fed from the time when they were two weeks' old for a period of 10 to 12 weeks on bread, cabbage and water, a diet practically free from fat: their livers yielded fatty acids the iodine value of which was 120. Again, young rats were fed for six weeks on bread and water and then for two weeks on bread, plasmon and water: the higher fatty acids from the livers of these animals had the mean iodine value of 152·7.

Conclusions.

1. The presence of the following higher fatty acids has been demonstrated in the liver.

(*a*) Palmitic acid, $C_{16}H_{32}O_2$.
(*b*) Stearic acid, $C_{18}H_{36}O_2$.
(*c*) Oleic acid, $C_{18}H_{34}O_2$.
(*d*) Linoleic acid, $C_{18}H_{32}O_2$.
(*e*) An acid having the formula $C_{20}H_{32}O_2$.

2. The occurrence of oleic acid in the liver has been proved by the isolation of dihydroxystearic acid from the products of oxidation. The properties of this substance have been described, and it has been shown that the oleic acid from which it is derived is different from other oleic acids. The double linkage in the liver oleic acid lies between the sixth and seventh carbon atoms, reckoning from the methyl-group end of the chain.

The double linkage in the oleic acid obtained from the adipose tissue is situated exactly in the middle of the chain.

3. The presence of linoleic acid has been proved by the preparation of a tetrahydroxystearic acid. Some evidence was obtained of the presence of two linoleic acids in the liver, as well as evidence as to the probable constitution of one of these.

4. The occurrence of the acid $C_{20}H_{32}O_2$ is shown by the formation of octobromoarachidic acid on bromination and octohydroxyarachidic acid on oxidation of the liver fatty acids.

5. Approximately one-half of the higher fatty acids of the liver are unsaturated acids. The acid $C_{20}H_{32}O_2$ occurs to the extent of about 10 % of the total fatty acids and linoleic acid is present in greater amount than oleic acid.

6. The high iodine values of the liver fatty acids, and also the ease with which they take up oxygen from the air, are due to the presence of linoleic acid and the acid $C_{20}H_{32}O_2$.

7. The unsaturated acids of the liver are present, to a considerable extent, in the form of lecithin and similar complex substances.

In conclusion, I desire to express my thanks to Dr J. B. Leathes, in whose laboratory this work has been carried out, for the interest he has taken in the investigation and for much valuable help and advice.

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ON THE WASSERMANN REACTION IN GENERAL PARALYSIS OF THE INSANE.*

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When the serum of a syphilitic patient is mixed with an extract made from the liver of a syphilitic fetus, the mixture possesses a property not possessed by either constituent alone in the same degree. It is able to take up complement. That this is the case is shown by adding to the mixture a suitable quantity of complement—that is, fresh guinea-pig serum—and after an interval adding sensitized red blood corpuscles. The red cells remain intact, suspended in the fluid; whereas, if the complement had not previously been taken up, they would have been laked.

A quite analogous phenomenon occurs in other conditions. If we mix the serum of a typhoid patient with an extract of typhoid bacilli, the mixture similarly acquires the property of fixing complement; and this is true for many other organisms and the corresponding serums. It seemed reasonable, therefore, to suppose, as Wassermann[1] did when he discovered the syphilis reaction, that in this case also he was dealing with an extract of the causal organism and the corresponding serum. In other words, the liver extract was active because it was an extract of the *Spirochaeta pallida* contained in the liver, and the serum was active because the patient had responded to the invasion of the spirochaete by producing an antibody in his blood. If this were so, the reaction would be specific; the complement would be fixed only if the serum came from a syphilitic, but would remain free if the serum were derived from a nonsyphilitic; and a method for the serum-diagnosis of syphilis would have been devised. This proved to be the case. The reaction is specific for syphilis, and its value as an aid to diagnosis is admitted. The theoretical support, however, and the convenient explanation of the specificity which the typhoid and other analogies supplied, have had to be abandoned. It has been shown by many workers[2 3 4] that the spirochaete is not an essential factor in the liver extracted. Non specific substances such as the heart of the guinea-pig,[5] or even combinations of such relatively well-defined bodies as lecithin, oleic acid, and sodium oleate,[6] may replace the syphilitic liver in the reaction. Its validity, therefore, as a means of diagnosing syphilis depends entirely on the empirical proof. This fortunately is present in embarrassing quantity. Wassermann[7] himself a year ago could report the examination of 1010 non-syphilitic serums without a single positive result, and although there is now a small number of cases on record in which a positive reaction has been obtained in the absence of syphilis, it is everywhere accepted that the reaction is thoroughly reliable as an aid to diagnosis.

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* The results of a considerable portion of this work have already been reported by Dr. Mott in his Morison Lectures. See British Medical Journal, 1909, February 20th, p. 454.

[352/09]

Immediately after his announcement of the method, Wassermann published with Plaut[8] a paper showing that if the cerebro-spinal fluid from cases of general paralysis of the insane were used in place of the syphilitic serum in his reaction, the complement was fixed in the same manner. This was hailed at once as supplying an objective proof of the connexion of general paralysis with syphilis, which had long been asserted on clinical grounds.

At Dr. Mott's request we undertook the examination of a series of spinal fluids from cases of general paralysis and other diseases, the result of which we record in this paper.

The method we have employed is essentially that of Wassermann. The blood corpuscles used to test for the presence of complement are derived from the ox, freed from serum by thorough washing in salt solution. They are sensitized by the serum (heated thirty minutes at 56° C.) of rabbits which had received several injections of washed ox corpuscles. As extract we used at first a watery extract from the liver of a syphilitic fetus prepared in the manner described by Wassermann; but later we have used an alcoholic extract made by extracting 1 gram of crushed and pounded liver with 4 c.cm. alcohol at room temperature for two days and then filtering through paper. Such an alcoholic extract remains unaltered in the cold for weeks, and is incomparably more convenient to work with. We have found no difference in the results obtained with the alcoholic as compared with the aqueous extract. Meyer[9] has stated that he obtains "10 per cent. finer results" with watery than with alcoholic extracts, but this has not been our experience. It can scarcely be the case for all livers, since only 25 per cent. of congenital syphilitic livers give an aqueous extract strong enough to be useful (as Georg Meier[10] has stated), and practically all such livers give excellent alcoholic extracts. Before use we dilute our extract, adding 4 parts of salt solution to 1 of extract; the salt solution should be added slowly, as was pointed out by Sachs and Rondoni.[11] As complement fresh guinea-pig serum was employed. The spinal fluid was heated for half an hour at 56° C. before use.

As the strength in complement of guinea-pig serum varies for each animal, and a fresh animal was used for each day's work, the dose of rabbit serum necessary to adequately sensitize the red cells is determined each day —that is, the smallest quantity of rabbit serum which totally laked the red cells in presence of the complement was ascertained; and for use in the actual examination of the spinal fluid two to three times this ascertained dose is taken. For each spinal fluid the tubes are arranged in two series of three tubes each, into which the doses of spinal fluid are measured, as in the example (see table at foot of page). To the first series are added the indicated doses of guinea-pig serum and liver extract, and finally salt solution to make the volume 2 c cm.; to the second series only guinea-pig serum and salt solution to 2 c.cm. A general control series of tubes is set up as shown. Then all tubes are placed at 37° C. for one hour, to allow fixation of the complement, if any, to occur. Then to all tubes, except the last three of the control series, is added the rabbit serum; and, finally, each tube receives 1 c.cm. of a 5 per cent. suspension of washed ox corpuscles in salt solution. The tubes are then shaken and placed for a further two hours at 37°. The results are then read off, and a second reading is made after the tubes have stood in the cold overnight.

The main difference in this method from that employed by Wassermann is that we have used ox blood corpuscles instead of sheep's. Human serum contains no, or inappreciable, amounts of amboceptor capable of sensitizing ox red cells. It does, however, usually contain amboceptor sensitizing sheep's red cells, and the amount of this may vary greatly with different individuals—for example, 0.25 c.cm. serum from one person may be insufficient to sensitize a quantity of cells, which is fully sensitized by 0.025 c.cm. from another.[12] [13] This uncertainty regarding the degree of sensitization of the red cells is troublesome when one is working with serums, and, although in examining spinal fluids it is not so significant, we preferred to use ox cells throughout our work.

We have examined 175 samples of cerebro-spinal fluid, derived from 127 individuals. Of these 127, 64 were cases of general paralysis, and in 59, or 92.1 per cent., a positive result was obtained, while in 5, or 7.8 per cent., the reaction was negative. These 64 cases include 7 from which the fluid was obtained only after death, and all of these gave positive results. It might be objected that the examination of such *post-mortem* fluids could give no trustworthy results. In the case of cerebro-spinal fluids, however (whatever may be the case with serums[14]), this is not so. If the fluid is withdrawn within twenty-four hours after death from a body which has lain in the cold room during the interval (as was done in all our *post-mortem* cases), the Wassermann reaction is not altered. Examinations were made of such fluids from over 30 control cases, with no positive results. The spinal fluid even of a general paralytic, which had been negative during life, remained so after death. And in every instance when we have been able to examine *post mortem** the fluid from a case positive during life, the reaction has remained the same. If withdrawn from the lateral ventricle *post mortem* the fluid is always clouded with fine amorphous flocculi, but if taken from the spinal canal it is clear and limpid as in life. In 22 cases fluids from both the spinal canal and lateral ventricle were examined, with in every case corresponding results; either both were positive or both negative. It is noticeable, however, that the cloudy fluid, while remaining sterile, deteriorates more quickly on keeping than the clear fluid from the spinal canal. Our percentage of positive results agrees well with those obtained by the more recent workers—for example, Marie, Levaditi, and Yamanouchi[15] obtained 93 per cent. positive in 30 cases, Plaut[16] 98 per cent. in 95 cases. The earlier work gave lower figures, Wassermann and Plaut[8] finding only 78 per cent. positive in 41 cases, and Marie and Levaditi[2] 74 per cent. in 39 cases. Of 352 cases collected from the literature, 309, or 87.7 per cent., give positive results.

Marie and Levaditi[2] pointed out that the percentage of positive reactions increased as the disease was more advanced. It is not easy to classify satisfactorily such a disease as general paralysis into definite stages, but, on the whole, our results agree with those of the French authors. Thus, of 21 cases undoubtedly in the most advanced stages of the disease, over 90 per cent. gave the reaction, while of 16 cases certainly in a middle stage, only 81 per cent. were positive. We examined also 9 cases (not included above) in which the definite diagnosis could not be made clinically, though it was considered possible that general paralysis might develop. These all gave negative results. It will be interesting to observe the further progress of these cases clinically and with regard to the Wassermann reactions. In 6 cases of advanced paralysis we tested the urine, with negative results. This agrees with the finding of Hoehne,[17] though Blumenthal and Wile[18] state that they have obtained positive results with urine in a large number of cases of syphilis.

Fluids from 63 cases not suffering from general paralysis have been examined, in 32 of which the fluid was obtained during life. In no single instance was a positive reaction obtained. These cases included melancholia (9), senile mania (10), epilepsy (2), cerebral tumour (2), cerebellar tumour, syphilitic meningitis, lues cerebri, tuberculous meningitis, alcoholism (3), disseminated sclerosis, dementia praecox, post-haemorrhagic and other softenings; and a few fluids received for diagnosis of tuberculous or epidemic meningitis.

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* Sixteen of our paralysis cases have died since our first examination of the fluid, the diagnosis in all cases being confirmed by the *post-mortem* findings.

	Se +
A. Spinal fluid known to be positive :—	
0.8 c.cm.	
0.4 c.cm.	
0.2 c.cm.	
0.1 c.cm.	
B. Spinal fluid from general paralysis :—	
0.8 c.cm.	
0.4 c.cm.	
0.2 c.cm.	
0.1 c.cm.	
C. Spinal fluid from general paralysis :—	
0.8 c cm.	
0.4 c.cm.	
0.2 c.cm.	N
0.1 c.cm.	
D. Spinal fluid from melancholia :—	
0.8 c.cm.	
0.4 c.cm.	
0.2 c.cm.	
0.1 c.cm	
E. General controls :—	
0.1 c.cm. guinea-pig serum + 0.2 c.cm. extract + salt so	
0.1 c.cm. guinea-pig serum + salt solution to 2 c.cm.	
*0.1 c.cm. guinea-pig serum + salt solution to 2 c.cm.	
*2 c.cm. salt solution	
*0.2 c.cm. extract + salt solution to 2 c.cm.	

* These tu

ig tract .cm.	0.1 c.cm. Guinea-pig Serum + Salt Solution to 2 c.cm.	Result.
	Total haemolysis	Positive.
	,,	
	,,	
	—	
	Total haemolysis	Strongly positive.
	,,	
	,,	
	—	
	Total haemolysis	Positive, but weak.
is	,,	
lysis	,,	
is	—	
	Total haemolysis	Negative.
is	,,	
	,,	
	—	

.. .. Total haemolysis.

... ... ,,

.. .. No haemolysis.

... . ,,

... ... ,,

---

bit serum.

In suspected general paralysis a positive Wassermann reaction with the cerebro-spinal fluid is practically diagnostic. In only two other conditions is it ever obtained,* namely, in tabes and in syphilis located in the central nervous system of the meninges. In tabes it occurs regularly, but with much less frequency than in general paralysis. Thus in 112 cases from the literature positive results were obtained in 57, or 50 per cent. In cerebral syphilis it is stated to occur occasionally, though exceptionally.[16, 19, 20] We have not yet met with a positive case, and it is evident that it is not the rule to obtain the reaction, although the serum may be strongly positive. These two diseases do not usually present difficulties in differential diagnosis, and apart from them the reaction is, so far as is yet known, decisive. It is unfortunate that it is precisely in the early stages of general paralysis, where diagnosis is most difficult, that the reaction most often fails us by giving a negative result. But even in the most advanced cases one occasionally obtains a clear negative. In one of our cases, two months before the patient died, the spinal fluid was negative, though his condition was such that he clearly had not long to live. Three days before death his serum was positive. The diagnosis was confirmed *post mortem*, macroscopically and microscopically. The spinal fluid was withdrawn two hours after death, and specimens of lateral ventricle fluid and of serum were obtained twenty hours after death, all of these giving very strong positive reactions. In this instance, then, although the fluid eventually became positive, it was still negative a short time before death, when the disease was far advanced. We have already referred to a case in which the fluid remained negative throughout.

	Cerebro-spinal Fluid.	Serum.
General paralysis:		
In literature ...	352 cases, 309 positive—that is, 87.7 per cent.	285 cases, 247 positive—that is, 86.6 per cent.
Our cases	64 cases, 59 positive—that is, 92.1 per cent.	10 cases, 9 positive—that is, 90.0 per cent.
Tabes dorsalis:		
In literature ...	112 cases, 57 positive—that is, 50.8 per cent.	176 cases, 125 positive—that is, 71.0 per cent.

In most cases of general paralysis not only the spinal fluid is positive, but also the serum. It has been said that the serum is invariably positive; but this is not the case. Plaut,[16] Lesser,[21] and Marinesco[22] have indeed recorded 100 per cent. of positive reactions in 169 cases; but Marie, Levaditi, and Yamanouchi[15] obtained only 59 per cent. positive in the serum (as against 93 per cent. in the spinal fluid), and others[23] have obtained even smaller figures. Of 285 cases in the literature, 86.6 per cent. gave positive reactions. We have been able to examine only 10 serums from paralytics, of which 9 were positive and 1 negative. These include a case in which the serum was positive but the spinal fluid negative. The occurrence of cases where the fluid is positive but the serum negative, and the fact that in ordinary syphilis the fluid is always negative though the serum is strongly positive, show that the existence of the reacting substance in the spinal fluid is not due to a mere filtration from the blood. The active body appears in the fluid as the result of a local production, due to an alteration in the nervous system itself or in its coverings. This alteration is of a specific character. The reaction does not always accompany the presence of excess of albumen in the fluid. Thus a positive reaction is not obtained in simple meningitis. Even in syphilitic meningitis we do not get a positive reaction. Through Dr. Mott's kindness we were able to examine such a case three times in two months, during which the cell contents fell under mercury from 350 cells in 1 c.cm. to 70, 20, and eventually none; all three examinations gave negative results. All such tests as depend on excess of globulin or albumen in the spinal fluid must, therefore, fail to be specific. Nor does a positive reaction necessarily accompany an excess of lymphocytes in the fluid (cf. Marie and Levaditi[2]). In 32 estimations of the presence of lymphocytes in the fluid of paralytics, we found these cells in marked excess in 31 and in slight excess in 1, but in only 29 was the reaction positive; while in our control series we have three times found the cells in excess, though the reaction was again negative. Further, the mere breaking up of nerve tissue, such as occurs in softening of the brain substance, whether post-haemorrhagic or otherwise, does not cause the fluid to react positively.

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* One case is recorded by Levaditi and Yamanouchi, where positive result was obtained with the lumbar fluid in a case of sleeping sickness.

We must assume that there is a highly specific nervous lesion, occurring almost exclusively in general paralysis and tabes, accompanied by the presence in the cerebrospinal fluid of a substance of a very special kind. It is usual to call this substance an antibody. There is, however, no ground for supposing that it is antibody in any ordinary sense of the term. Since the typhoid and other similar analogies have been abandoned, there is no valid reason to suppose that the substance is directly connected with the spirochaete in any such immediate relation as subsists between a typhoid or diphtheria antibody and its bacillus or toxin. So far as we know at present, it is merely a symptom, like the sugar in a diabetic urine. Its presence confers no immunity. It is normally found in the serum of some of the lower apes,[24] which can be readily infected with syphilis, and the infectivity of active virus is not neutralized *in vitro* by a serum rich in "antibody." In man its presence in the blood is usually taken to indicate that he is still the subject of active syphilis, which clinically may be entirely latent; and Citron[25] goes so far as to formulate this into a so-called "law," "a positive reaction denotes active syphilis." As a general statement, however, this cannot be correct. Tabes and general paralysis can scarcely be described as active syphilis, yet, as we have seen, the serum in these diseases gives a positive reaction in 70 to 90 per cent. of cases. In the spinal fluid it may be held even to be injurious rather than beneficial, since it has a definite affinity for so important a constituent of normal nervous tissue as lecithin. Further, its occurrence in the serum and spinal fluid of general paralysis and tabes, while it adds another argument to the clinical evidence connecting these diseases with syphilis, in no sense proves that connexion incontestably.

We have pleasure in thanking Dr. Mott for the interest he has taken in our work, and the assistance he has rendered us throughout. We also acknowledge with pleasure the kind assistance given us in obtaining material by Dr. Robert Jones, the Medical Superintendent, and Dr. Hughes, Medical Officer of the London County Asylum at Claybury; Dr. Bond, Medical Superintendent of Long Grove Asylum; Dr. Ingram and other medical officers of the London infirmaries; Mr. Gibbs, Surgeon at the London Lock Hospital; and Dr. Nepean Longridge, of Queen Charlotte's Lying-in Hospital for Women.

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ON THE ESTIMATION OF PURINE BASES IN URINE.

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In the course of some work on the subject of endogenous uric acid (1), it was found that the figures obtained by the Camerer-Arnstein method for the total purine nitrogen of urine were in many cases lower than those representing the nitrogen of the uric acid alone; in the remainder there was great irregularity, the total purine nitrogen often exceeding the uric acid nitrogen by but a very small amount. In the series of observations published by Macleod and Haskins (2), who employed the same methods of estimation, the total purine nitrogen is on some occasions but slightly greater than the uric acid nitrogen, and on two occasions is lower than it.

At Dr Leathes's suggestion, some experiments were carried out to test the accuracy of the Camerer-Arnstein method.

THE CAMERER-ARNSTEIN METHOD.

The analysis is carried out in the following way (3).

The phosphates of the urine are removed by means of magnesia mixture and ammonia. Ammoniacal silver nitrate is added to the filtrate; the precipitate is filtered off by means of the pump, the last portions being washed on to the filter with weak ammoniacal water. The precipitate is then washed with water until the filtrate is neutral to litmus paper. The precipitate and filter paper are then transferred to a Kjeldahl flask, and boiled with water and magnesium oxide till very little water is left (Neubauer and Vogel, *Analyse des Harns*, 10th ed. p. 817). The amount of nitrogen present is estimated by the Kjeldahl process. In the method as described originally (3) the precipitate was not boiled before the Kjeldahl estimation. Salkowski (4) pointed out that these precipitates contain ammonia even after prolonged washing. Subsequently Arnstein recommended boiling with magnesium oxide as a means of removing this ammonia, and stated that he obtained the correct amounts of nitrogen when testing the method, as thus modified, with known weights of uric acid (5). He does not bring forward these results.

(a) *Results obtained with uric acid.*

As uric acid is the purine present in largest amount in urine, and as seemed to be the most likely to undergo decomposition when its lver compound was boiled, it was used in the first experiments.

(1) Weighed amounts of uric acid were dissolved with caustic soda. Acetic acid was lded until the uric acid began to be precipitated. Ammonia was then added until mplete solution was obtained; by this means, the presence of an excess of caustic soda as avoided. The uric acid was precipitated with magnesia mixture and ammoniacal lver nitrate; in order to secure as complete precipitation as possible the amounts of lver and magnesium added were about three times as great as those calculated to be ecessary[1]. The mixture was in all cases allowed to stand overnight before filtering. o remove the ammonia, the precipitates were washed till the filtrates were neutral and ee from chlorides and nitrates, and were then dried for five hours at 65° C. The pump as not used in filtering.

	Calculated	Found	Loss per cent.	
nboiled ...	22·90 mg. N	20·93	8·6	Mean 4·9
	26·66	25·92	2·8	
	22·27	21·56	3·2	
oiled 2 hours ...	25·93	22·26	14·2	Mean 13·2
	31·90	25·67	19·5	
oiled 2 hours with MgO	13·57	11·48	15·2	
	24·43	22·47	8·1	
	35·40	32·13	9·0	
oiled nearly to dryness with MgO	21·70	18·76	13·5	
	24·53	21·65	11·7	

(2) Two precipitates were prepared as described above, but were not dried before iling. Arnstein's directions as regards amount of nitrogen, time of standing before tration, method of filtration, and time of boiling, were followed.

Calculated	Found	Loss per cent.
15·07 mg. N	12·95	14
11·33	9·73	14

(3) A number of experiments were carried out in a similar manner with silver urate, magnesia mixture being used; about the same average loss (13 °/₀) occurred in the iled preparations.

No relation could be observed between the duration of boiling and the amount of loss.

Duration of boiling	Calculated	Found	Loss per cent.
15′	29·40 mg. N	24·94	15·2
30′	33·33	29·61	11·2
60′	27·53	23·74	13·8

(4) A number of estimations were carried out in which silver urate or silver magnesium ate was boiled with magnesium oxide, the mixture then treated with sodium or hydrogen

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[1] When precipitated by these solutions, uric acid is combined in the proportion of one olecule to one atom of each of the two metals (Neubauer and Vogel, *op. cit.* p. 318).

sulphide, and the uric acid present estimated gravimetrically as in the Salkowski-Ludwig process. The boiled preparations, in comparison with the controls, showed a loss of about 40 $^0/_0$ of the weighed amount of uric acid present at the outset. This method could not, of course, demonstrate that any loss of nitrogen occurs in boiling.

These experiments indicated that the low results obtained by the Camerer-Arnstein method were due chiefly to decomposition of uric acid during boiling. Accordingly, in further experiments, the influence of boiling was eliminated.

(5) The precipitate when washed neutral was placed in a combustion flask with magnesium oxide and kept at 37° C. and 30 mm. Hg pressure till apparently dry.

In one case, this dry residue was extracted with water, and the amount of ammonia present estimated by Nessler's reagent; 0·05 mg. ammonia was found.

	Calculated	Found	Loss per cent.	
(*a*)	26·10 mg. N	25·27	3·2	Mean 4·9
(*b*)	22·67	20·72	8·6	
(*c*)	25·03	23·77	5·0	
(*d*) *In vacuo* when all on filter. Not washed neutral ...	20·73	20·16	2·8	

The average loss in these cases is the same as in those described above (p. 297) in which the precipitates were not boiled. Since the method here employed to remove ammonia was shown to be effectual, the higher result of estimation (*d*) is probably due to the absence of prolonged washing with consequent solution of some of the precipitate.

It was thought possible that there might be some evolution of free nitrogen during boiling, owing to the action upon the uric acid of nitrite formed from the silver nitrate by reduction. Magnesium silver urate was therefore boiled with magnesium oxide in a stream of CO_2 which passed into a nitrometer filled with potash. No nitrogen was obtained. Moreover, subsequent experiments showed that the ammonia-nitrogen given off during boiling gave with the nitrogen found in the precipitate and filtrate a total which was approximately equal to the amount of nitrogen present at the outset.

If the filtrates from the precipitate of magnesium silver urate be kept for a few days, a further quantity of the flocculent white precipitate generally appears in them. An attempt was made therefore to estimate the fractions of the total loss due respectively to incomplete precipitation and to decomposition during boiling. With this object the ammonia contained in the precipitate was by various means removed as completely as possible; the precipitate was then boiled, and the distillate received in $\frac{N}{10}\ H_2SO_4$. In some cases the filtrate was kept for two or more days, the precipitate in it then filtered off, and together with the filter paper boiled with magnesium oxide to remove ammonia; the nitrogen present was then estimated by the Kjeldahl process.

(6) Uric acid dissolved with sodium carbonate.

Three portions of the solution precipitated with magnesium chloride and ammoniacal silver nitrate, precipitates washed neutral to litmus, placed in a vacuum desiccator over H_2SO_4 at 37° for 10 days, re-exhausted twice. At the second exhaustion, a piece of moist itmus paper was placed in the desiccator, and an evolution of ammonia observed by this means; at the third exhaustion, this test gave a negative result. Precipitates boiled with MgO for 1½ hours.

Precipitates	Distillate	
89·60 mg. N	4·40	(1st 30′)
	0·75	(2nd 30′)
	0·65	(3rd 30′)
	5·80	mg. N = 6·1 $^0/_0$ of 89·60 + 5·80.

A portion of another solution was treated in the same way as described above, and oiled with MgO for two hours.

Precipitate	Distillate	
28·28 mg. N	2·28	(1st hour)
	0·46	(2nd hour)
	2·74 mg. N = 8·8 $^0/_0$ of 28·28 + 2·74.	

(7) Uric acid dissolved with sodium carbonate.

$$\text{Solution, Kjeldahl}\begin{cases}33\cdot01 \text{ mg. N in 100 c.c.}\\ 33\cdot15\end{cases}$$

Precipitated with magnesium chloride and ammoniacal silver nitrate. Precipitate ashed neutral to litmus, and suspended in water in a combustion flask; the flask was ept at 35° C., and a current of air passed through the liquid. The ammonia which came ver was received in $\frac{N}{10}$ H_2SO_4, which was titrated at intervals. After 6 hours, no more mmonia was obtained. The precipitate was then boiled with magnesium oxide for two ours.

Precipitate		Filtrate	Distillate	
28·70	+	1·54	1·62	(1st hour)
			0·60	(2nd hour)
			+ 2·22 = 32·46	

(2·22 = 7·2 $^0/_0$ of 28·70 + 2·22).

Ammonia is therefore formed during boiling. This can be shown ore satisfactorily if no ammoniacal reagents be used in the preparation f the precipitate. In the following experiments, magnesium sulphate nd silver nitrate were used for precipitation.

(8) 1·25 gr. uric acid was mixed with enough 2 $^0/_0$ caustic soda to convert 1 gr. uric id to acid sodium urate. The mixture was left overnight, filtered, and made up to 500 c.c. ne results showed that 0·849 gr. uric acid had dissolved. By this means, the presence of

a large excess of soda was avoided; the washings from the precipitate were neutral. Any caustic soda remaining adherent to the precipitate would have caused destruction of uric acid during boiling. Sodium carbonate cannot be used if silver nitrate is to be added.

Solution of acid sodium urate.

A. 50 c.c. Kjeldahl $\begin{cases} 27·58 \text{ mg. N} \\ 27·58 \end{cases}$

B. 50 c.c. precipitated with $MgSO_4$ and $AgNO_3$.

		Precipitate		Filtrate	Distillate		
(*a*)	... ...	26·95	+	0·63			=27·58
(*b*)	Boiled 2 hours with MgO	25·27	+	0·35	0·91	(1st hour)	
					0·91	(2nd hour)	
					+1·82		=27·44

(1·82=6·7 % of 25·27+1·82).

(*c*)	Boiled 1½ hours with MgO	25·69	+	0·63	0·84	(1st 30′)	
					0·70	(2nd 30′)	
					0·49	(3rd 30′)	
					+2·03		=28·35

(2·03=7·3 % of 25·69+2·03).

Estimation (*a*) above indicates that if prolonged washing, and boiling, be avoided, the only source of error in this method is incomplete precipitation.

It was thought possible that the presence of small amounts of ammonia, such as are present when the method is used in the ordinary way, might facilitate the decomposition of the silver compound. Accordingly, in estimations (*b*) and (*c*) above, known amounts of a very weak titrated solution of ammonia were added before the final period of boiling, and these amounts subtracted from those found in the distillate. The percentage loss is, however, about the same as on other occasions.

The results given below afford further evidence of the increased accuracy of the method when boiling is avoided. A blank nitrogen estimation made upon the amounts of magnesium sulphate and silver nitrate present in these filtrates yielded the same figure that was used throughout this investigation (0·3 c.c. $\frac{N}{10}$ NH_3).

(9) Uric acid dissolved with caustic soda as acid sodium urate, as in (8) above. Precipitation with $MgSO_4$ and $AgNO_3$.

Solution 1. Kjeldahl. $\begin{cases} 31·36 \text{ mg. N in 100 c.c.} \\ 31·36 \end{cases}$

Precipitate		Filtrate		
31·22	+	0·35	=	31·57
31·22	+	0·70	=	31·92
31·15	+	0·49	=	31·64

Solution 2. Kjeldahl. $\begin{cases}30{\cdot}31\\30{\cdot}31\end{cases}$

Precipitate		Filtrate		
29·12	+	0·91	=	30·03
29·05	+	0·91	=	29·96
29·19	+	0·98	=	30·17

The experiments described above showed that when magnesium silver urate is boiled a loss of nitrogen occurs which amounts to from 6 to 9 $^0/_0$. This loss does not occur unless the uric acid be combined with the metals. Berthelot and André⁽⁶⁾ found that uric acid gave off no ammonia when boiled for an hour with magnesium oxide. In the present series, an experiment was made in which 0·2286 gr. uric acid was boiled for 75 minutes with silver hydrate and magnesium oxide; the amount of ammonia obtained was equivalent to only 0·8 $^0/_0$ of the nitrogen of the uric acid.

(*b*) *Results obtained with purine bases.*

It was now necessary to ascertain whether the silver compounds of purine bases[1] undergo this loss during boiling.

(1) *Hypoxanthin, ammoniacal solution.* Two portions precipitated with ammoniacal silver nitrate; precipitates washed till neutral and free from chlorides and nitrates.

(*a*) Kjeldahl, 17·92 mg. N.
(*b*) Boiled two hours with MgO, 17·64.

(2) *Hypoxanthin.* (Contained 40·69 $^0/_0$ nitrogen, calculated 41·18 $^0/_0$.) Two weighed amounts dissolved with ammonia, precipitated with ammoniacal silver nitrate, washed till washings gave very slight Nessler reaction.

	Calculated	Found
(*a*) Kjeldahl	39·75 mg. N	39·55
(*b*) Boiled two hours with MgO	64·61	63·98

(3) *Purine bases of urine.* The phosphates and uric acid of urine were precipitated with magnesia mixture, ammonia, and ammonium sulphate. The filtrate was precipitated with ammoniacal silver nitrate. The precipitate was washed on a number of small filters until 50 c.c. of the mixed filtrates contained 0·07 mg. ammonia, and was then decomposed with H_2S. Portions of the solution were precipitated with silver nitrate, no ammonia being used. In some cases the precipitation and filtration were carried out in a dark room. The filtrates from each pair of precipitates were mixed, and the nitrogen present estimated. The distillate from the precipitates which were boiled was received in $\frac{N}{10}H_2SO_4$.

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[1] Purine bases when precipitated by ammoniacal silver nitrate combine with a group Ag_2O (Neubauer and Vogel, *Analyse des Harns*, 10th ed. p. 340). No statement is made here as to combination with magnesium.

(A) Solution. Kjeldahl. $\left.\begin{array}{l}20\cdot14\\19\cdot79\end{array}\right\}$ 19·97 mg. N

Silver nitrate precipitates:

		Precipitate		Filtrate	
(B)	... ...	18·16 18·44	+	1·54	=19·70 =19·98
(C)	Boiled 25′ with MgO	18·23 19·01	+	1·73	=19·96 =20·74
(D)	... ... One lost.	19·36	+	0·49	=19·85
(E)	Boiled 25′ with MgO	18·87 18·87	+	0·78	=19·65 =19·65

(D), (E). Kept in dark room.

(C), (E). No ammonia evolved during boiling.

The silver compounds of the purine bases which were examined did not therefore undergo the decomposition during boiling which occurs in the case of uric acid.

A MODIFICATION OF THE CAMERER-ARNSTEIN METHOD.

Since uric acid was found to undergo loss when treated by the Camerer-Arnstein method, while the purine bases which were examined were not found to undergo this loss, the Camerer-Arnstein procedure was employed for the estimation of purine bases in urine after the removal of the uric acid.

Method A.

This method has been used in three series of observations (Cathcart, Kennaway, and Leathes, *Quart. Journ. Med.* I. p. 416, Protocol X.; Kennaway, *Journ. of Physiol.* XXXVIII. 1).

The estimation of uric acid is carried out by the method of Folin and Schaffer(7); the ammonia added must be measured accurately, and the mixture kept during the precipitation in a closed vessel to prevent evaporation.

If 100 c.c. of urine be taken for the estimation, 100 c.c. of the filtrate obtained after the addition of the ammonium sulphate solution is placed in a 200 c.c. Erlenmeyer flask. 10 c.c. of 15 °/₀ ammonia is added. When the ammonium urate is filtered off, 100 c.c. of the filtrate is taken for the estimation of purine bases. To this are added 10 c.c. of 20 °/₀ ammonia and 10 c.c. of magnesia mixture. The phosphates are filtered off, and 10 c.c. ammoniacal silver nitrate added to 100 c.c. of the filtrate; the mixture is allowed to stand overnight, and the estimation completed as in the Camerer-Arnstein method.

The 100 c.c. of filtrate to which the silver nitrate is added represents $\frac{4}{5}$ of $\frac{10}{11}$ of $\frac{10}{12}$ of 100 c.c. of urine, that is, 60·6 c.c. of urine. It contains $\frac{10}{12}$ of the 3 mg. of uric acid stated by Folin and Schaffer to escape precipitation. From the amount of nitrogen found, the nitrogen of this uric acid, namely 0·83 mg., must be subtracted; the resulting figure, multiplied by $\frac{100}{60\cdot6}$, or 1·65, gives the amount of purine base nitrogen present in 100 c.c. of urine.

The precipitation of any of the purine bases together with the uric acid would of course introduce an error into this method. Hopkins(8) found that xanthin was precipitated by saturation of an ammoniacal solution with ammonium chloride. With a mixture of uric acid and a small proportion of xanthin, the latter was precipitated to a large extent by saturation with ammonium chloride, but the whole of it was found in solution when the uric acid was liberated. Hypoxanthin was not precipitated in this way.

In Beilstein's *Hdb. d. organ. Chemie* it is stated (B. III. S. 962) that xanthin dissolved by means of potash is not precipitated by ammonium chloride.

Edmunds(11) found that saturation of solutions of hypoxanthin and xanthin with ammonium sulphate produced no precipitate.

Folin(9) could not observe any precipitation of xanthin by ammonium chloride. He found guanin to be precipitated by ammonium chloride, carbonate, and sulphate, but not by the acetate[1].

This source of error in method A was examined as follows.

A solution of purine bases was prepared from urine. The phosphates of the urine were removed with ammonia and barium nitrate. The purines were precipitated with ammoniacal silver nitrate; the precipitate was washed and decomposed with hydrogen sulphide. The filtrate was evaporated to a small volume after addition of hydrochloric acid, and the uric acid filtered off. The filtrate was precipitated with silver nitrate, and the processes of decomposition and evaporation carried out as before; after this second evaporation, no separation of uric acid could be observed.

The amount of purine base nitrogen in the solution was ascertained by precipitation with ammoniacal silver nitrate. Other portions of the solution were treated as follows: (*a*) Folin and Schaffer's ammonium sulphate solution, and ammonia, were added in the proportions used in uric acid estimations, (*b*) ammonium sulphate solution and ammonia were added in amounts producing a concentration of 10 °/₀ of ammonium sulphate in the whole mixture. In both cases, the mixtures stood overnight and were then filtered. The purine bases in measured fractions of the filtrates were precipitated with ammoniacal silver nitrate; the filter papers were boiled with magnesium oxide, and the nitrogen present estimated. The following results were obtained.

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[1] This precipitation of purine bases by ammonium salts might introduce an error into Wörner's method for the estimation of uric acid (*Zeit. f. physiol. Chem.* XXIX. p. 70. 1900). Wörner concludes that no such precipitation occurs, but he offers no evidence in support of this conclusion.

	Purine base solution		Precipitate		Total
(1) Original solution	6·89 mg. N 6·79 6·72	Mean 6·79			
(2) After treatment with Folin and Schaffer's solution (one lost) ...	6·12	+	0·28	=	6·40
(3) After treatment with ammonium sulphate 10 %.	6·27	+	0·42	=	6·69
	6·13	+	0·63	=	6·76

These results indicated that about 10 % of the purine bases was precipitated by the solutions used. It was thought that this result might be due to the presence of some uric acid in the purine base solution; accordingly, a similar experiment was carried out upon a portion of the solution which had been treated with manganese dioxide in the manner employed by Krüger and Schmid for the oxidation of uric acid (Hoppe-Seyler and Thierfelder's *Hdb.* 7th ed. p. 436).

	Purine base solution after oxidation		Precipitate		Total
(1) Original solution	4·41 mg. N.				
(2) After treatment with Folin and Schaffer's solution.	3·82	+	0·59	=	4·41
	3·79	+	0·56	=	4·35

The loss here was even greater than it had been before oxidation.

It appeared then that the figures obtained by method *A* for the purine bases must be too low; the method described below was designed to avoid the source of error.

Method B.

In Hopkins's method of uric acid estimation, any purine bases precipitated by the ammonium salt should be in solution in the acid mother-liquor from which the uric acid separates. Attempts were made therefore to employ this method in the estimation of purine bases.

The results obtained by precipitating known weights of uric acid with ammonium chloride were not satisfactory. Moreover, it was observed later that ammoniacal silver nitrate does not precipitate the purine bases in urine saturated or half-saturated with ammonium chloride[1].

In the following experiments, the 50 % ammonium sulphate solution of Folin and Schaffer (7) was used.

(1) Uric acid dissolved with sodium carbonate, made up to 500 c.c.

(*a*) 150 c.c. Kjeldahl. 68·31, 68·31 } mg. uric acid in 150 c.c. = 22·77 mg. in 50 c.c.

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[1] This result suggests that ammoniacal silver nitrate cannot be used to demonstrate the complete precipitation of uric acid by ammonium chloride.

(*b*) 50 c.c. + 50 c.c. water + 25 c.c. Folin-Schaffer solution + 5 c.c. ammonia, stood overnight. Precipitates washed twice with 10 % ammonium sulphate and titrated.

22·50 mg.	Mean 22·84 mg. in 50 c.c.
22·71	
22·87	
23·25	

(2) Weighed amounts of uric acid dissolved with sodium carbonate in 100 c.c. water. 25 c.c. Folin-Schaffer solution and 5 c.c. ammonia added. Stood overnight. Precipitates washed once with 10 % ammonium sulphate and titrated.

Calculated	Found
49·70 mg.	48·08
30·80	30·45

These results indicate that the correction given by Folin and Schaffer (+3 mg. for 100 c.c. urine) does not apply to pure solutions of uric acid.

Since ammonium sulphate was found to give fairly satisfactory results with uric acid alone, this salt was used in the separation of uric acid and purine bases.

In the uric acid estimations Hopkins's method of procedure was followed, except that ammonium sulphate in 10 % strength was used, and the mixture allowed to stand overnight as in the Folin-Schaffer process, in which the same proportion of sulphate is present. The urate precipitates were washed twice with 10 % ammonium sulphate. No correction was used for the sulphate mother-liquor. For the acid mother-liquor Hopkins's correction was used (+1 mg. for 15 c.c.).

The purine base solutions used were prepared from urine, as described above (p. 303). The filtrates and washings obtained from the precipitates of ammonium urate and uric acid were united, and precipitated with ammoniacal silver nitrate, the concentration of silver being the same as in the control estimations upon the purine base solution. The nitrogen of the amount of uric acid reckoned to be present in the acid mother-liquor was subtracted from the result of the purine base estimation.

It will be seen that in these estimations the purine bases show no tendency to keep the uric acid in solution.

This method has been applied to urine in the following way.

10 grs. ammonium sulphate are placed in a beaker. 100 c.c. urine and 5 c.c. strong ammonia are added, and the mixture stirred till the salt is dissolved. Next day the precipitate is filtered off, the last portions being washed on to the filter with 10 % ammonium sulphate. The filtrate and washings are received in a 250 c.c. measuring flask. The uric acid estimation is completed by Hopkins's method; less than 10 c.c. of water is required for the removal of chlorides. The acid filtrate and washings are received in a measuring cylinder, and transferred to the 250 c.c. flask. To the mixture in the flask are added 10 c.c. 20 % ammonia and 10 c.c. magnesia mixture; the flask is then filled up to the mark, and the contents filtered[1]. To 240 c.c. of the filtrate 20 c.c. ammoniacal silver

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[1] In methods A and B, it is important to shake the mixture thoroughly before filtering off the phosphates. Berthelot and André(10) found that ammonium magnesium phosphate was not decomposed appreciably by boiling for an hour with magnesium oxide.

Analyses of mixtures of uric acid and purine bases.

		Series I. 0·2584 gr. uric acid dissolved with ammonia in 500 c.c. water. Found Titration+Correction	Series I. Calculated	Series II. 0·2558 gr. uric acid dissolved with ammonia in 500 c.c. water. Found Titration+Correction	Series II. Calculated
Uric Acid.					
(1) 100 c.c. uric acid solution +13 grs. $(NH_4)_2SO_4$ +5 c.c. ammonia	+ 25 c.c. water	51·18	51·68 mg. uric acid	50·20	51·16
(2)	+ 25 c.c. water	50·43		49·45	
(3)	+25 c.c. purine base solution	50·61		50·20	
(4)	+25 c.c. purine base solution	51·55		49·80	
Purine Bases.					
Solution, 25 c.c.		5·60 5·53	Mean 5·57 mg. N	8·47 8·33	Mean 8·40 mg. N
Recovered from mixture with uric acid ...	(3)	6·44		8·61	
	(4)	6·30		8·68	
Subtract uric acid nitrogen in accordance with correction (0·93 mg. in each case)	(3)	5·51		Subtract 0·7 mg. 7·91	
	(4)	5·37		,, 0·6 mg. 8·08	

Total Purine Nitrogen.

		U.A.N.	+	P.B.N.	=	T.P.N.	U.A.N.	+	P.B.N.	=	T.P.N.
Calculated		17·23	+	5·57	=	22·80 mg. N	17·05	+	8·40	=	25·45
Found	(3)	16·87	+	5·51	=	22·38	16·73	+	7·91	=	24·64
	(4)	17·18	+	5·37	=	22·55	16·60	+	8·08	=	24·68

nitrate is added, and the mixture left overnight. The estimation is completed by the Camerer-Arnstein method[1]. The following corrections have been used: (1) +3 mg. uric acid for 100 c.c. urine, as in the Folin-Schaffer method, and (2) +1 mg. uric acid for 15 c.c. acid mother-liquor, as in Hopkins's method. To the result of the purine base estimation $\frac{1}{24}$ is added. The amount of nitrogen which was present as uric acid is subtracted, in accordance with the above corrections. The figure obtained is the amount of purine base nitrogen in 100 c.c. urine.

Parallel estimations by methods A and B have been carried out upon several samples of urine, 100 c.c. being taken for each estimation. In series 1 and 2, 25 c.c. of 50 % ammonium sulphate was used in the estimations by method B in place of the solid salt, and the correction increased accordingly (+ 3·75 mg.).

Analyses of urine.

		Uric acid mg. in 100 c.c.	Purine base nitrogen mg. in 100 c.c.	Total purine nitrogen in 100 c.c. urine					
1	A	34·88	2·26	11·63	+	2·26	=	13·89	13·75
		34·88	1·98	11·63	+	1·98	=	13·61	
	B	36·49	2·31	12·16	+	2·31	=	14·47	14·54
		36·49	2·45	12·16	+	2·45	=	14·61	
2	A	37·23	7·40	12·41	+	7·40	=	19·81	
		36·99	lost						
	B	38·57	6·77	12·86	+	6·77	=	19·63	19·77
		38·37	7·12	12·79	+	7·12	=	19·91	
3	A	34·18	3·61	11·39	+	3·61	=	15·00	14·59
		33·94	2·87	11·31	+	2·87	=	14·18	
	B	35·37	3·07	11·79	+	3·07	=	14·86	14·80
		35·30	2·97	11·77	+	2·97	=	14·74	
4	A	28·08	2·90	9·36	+	2·90	=	12·26	12·35
		27·61	3·25	9·20	+	3·25	=	12·45	
	B	26·35	3·57	8·78	+	3·57	=	12·35	12·36
		27·48	3·21	9·16	+	3·21	=	12·37	
5	A	28·08	1·63	9·36	+	1·63	=	10·99	11·03
		28·31	1·63	9·44	+	1·63	=	11·07	
	B	28·05	2·36	9·35	+	2·36	=	11·71	11·74
		28·06	2·41	9·35	+	2·41	=	11·76	
Averages of above figures:									
	A			10·81	+	3·50	=	14·31	
	B			11·02	+	3·62	=	14·64	
6	A	35·58	6·14	11·86	+	6·14	=	18·00	18·10
		36·51	6·02	12·17	+	6·02	=	18·19	
	B	41·19	4·75	13·73	+	4·75	=	18·48	18·37
	(H_2SO_4)	41·17	4·55	13·72	+	4·55	=	18·27	

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[1] In all the estimations which have been made by methods A and B, the filtration of the silver compounds has been carried out on an unfolded 9 cm. paper without the use of the pump.

A comparison of the results obtained by the two methods shows that method B gives on an average somewhat higher figures. It had been expected that this method would give still higher figures for the purine bases, as the method was adapted to recover any purine bases precipitated by the ammonium salt, and in the experiments which were made with reference to this (p. 303) about 10 % of the bases was so precipitated. The fact that the two methods when applied to urine give average results for the purine bases differing by much less than 10 % may be due to the following reasons.

(1) The purine bases of urine are probably precipitated by salts more readily after they have been separated from urine, as is the case with uric acid (p. 305). The experiments described above (p. 303) which were carried out upon purified bases, and which led to the employment of method B, may therefore indicate a degree of error in method A which is not present in analyses of urine. Moreover, the proportion of the purine base or bases in urine precipitable by ammonium sulphate will not be constant, and this proportion may have been high in the urine from which the purified bases were obtained.

(2) During the first two filtrations in method A there is considerable opportunity for evaporation, which will tend to raise the figure obtained for the purine bases. In B, evaporation could affect the result only during the final removal of the phosphates, which is effected very rapidly.

(3) Method B requires the higher correction for unprecipitated uric acid. Such corrections do not pretend to a high degree of accuracy, and their error will have a marked effect in one or other direction upon the figures obtained for the small proportion of purine bases.

In the analyses of urine given on p. 307 it is seen that, in each of the six series, with the exception of the first, the method giving the higher result for the uric acid gives the lower result for the purine bases. This is especially evident in series 6; in this case, in method B, sulphuric acid (4 drops) was used in place of hydrochloric acid for the decomposition of the ammonium urate. Hence the figures obtained by the two methods for the total purines agree together more closely than do those for either the uric acid nitrogen or purine base nitrogen. The explanation of this would appear to be that the amounts of unprecipitated uric acid are variable, and these amounts will be recovered in the purine base estimation.

The amount of purine base nitrogen in 100 c.c. of urine being so small, the estimation of this amount is naturally subject to considerable error. This is seen in the discrepancy between some of the duplicate results given on p. 307.

The error due to the process of analysis, as distinct from that due to the use of somewhat arbitrary corrections, may of course be lessened by using larger amounts. Several portions of 100 c.c. of urine may be prepared in the manner described above; two of these are used for the estimation of uric acid; in the others the amount of uric acid obtained is not determined, but all the filtrates are used for the estimation of purine bases.

The methods put forward here have an advantage in that the estimations of uric acid and purine bases are carried out upon the same sample of urine, whereby an economy is effected which is of importance in experiments in which the urine is analysed several times daily. Method A has advantages over method B in that the analyses are completed in a shorter time, and the process of uric acid estimation is simpler.

SUMMARY.

Uric acid, when treated by the Camerer-Arnstein method for the estimation of the total purines of urine, loses nitrogen in the form of ammonia. The formation of ammonia occurs when the precipitate of silver-magnesium urate is boiled.

The purine bases which were examined were found not to undergo this loss.

The Camerer-Arnstein method can be used for the estimation of the purine bases in urine from which the uric acid has been removed, but is subject to considerable error when small quantities only of urine are dealt with.

In conclusion, I wish to acknowledge my indebtedness to Dr Leathes and Dr Aders Plimmer for advice in carrying out this work, which was done during the tenure of a Research Scholarship of the British Medical Association.

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OBSERVATIONS ON THE INFLUENCE OF HEATING UPON THE NUTRIENT VALUE OF MILK AS AN EXCLUSIVE DIET FOR YOUNG ANIMALS.

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(1 Figure.)

During the year 1904, a year which included four months of hot summer weather, the mortality among infants under one year old in England and Wales was 145·3 per 1000 births. Of this mortality 21·8 % died of diarrhoeal diseases and 31·5 % from wasting diseases.

Experience shows that these troubles affect hand-fed to a much greater extent than breastfed infants.

Thus, in Paris, in 1898, from the 14th to the 27th of August 550 children died of diarrhoea. Of these 57 are recorded as being breast fed and 493 as artificially fed (Budin, 1907).

In Rouen during 1900 to 1905 out of 550 babies attending a large dispensary 57 % were breast fed (1), 7·4 % were on breast and artificial feeding (2), 35·6 % on artificial feeding only (3). Of these 7·6 % of (1) died, 26 % of (2) and 35·6 % of (3) (Budin, 1907).

Similar statistics have been published for Lille, Elboeuf and other places.

There are no available statistics of any large number of babies in England, but in Finsbury out of 212 infant deaths 21 were due to diarrhoea; of these none were breast fed, 4 were on breast and other foods and 17 were artificially fed (Finsbury Report on Public Health for 1907).

A large proportion of this infantile mortality must be attributed to a harmful or faulty nutriment, and as the diet of children of less than one year old is practically confined to milk, the remedy consists in securing for the infants innocuous milk of adequate nutritive value.

Much of this continual waste of infant life, is no doubt due to bacterial contamination of the milk, a danger which can be nullified by the heating of the milk shortly before its use. Provided that the temperature is sufficiently high and the length of time it is maintained adequate, the destruction of micro-organisms can be ensured, but, to effectively reduce the mortality from infected milk the remedy must be one which can be carried out in the poorest tenement lodging, that is, one which demands a minimum of skill and necessitates no special apparatus.

The raising of milk to the boiling point and allowing it to cool with some sort of covering fulfils these conditions. Why is this simple precautionary measure not put into practice? The reason is, I believe, due to a widespread belief in this country that the boiling of milk deprives it of some properties essential to the infant's well-being. Many medical men are convinced that rickets, scurvy and other troubles follow the use of boiled milk. Infantile scurvy seems, however, to be an uncommon disease and not unusually has been caused by feeding on some patent farinaceous baby-food.

In Paris, where several thousand babies are fed every year on milk that has been boiled for 20 minutes, rickets is rare and scurvy almost unknown; thus Budin (1907, p. 198) has not seen a single case of scurvy among children fed on such milk, and his experience is borne out by Bresset (1888—1905) who has one of the largest milk dispensaries in Paris.

During recent years a mass of valuable evidence has been accumulated in France showing that a diet of milk which has been boiled for 45 minutes gives excellent results.

Budin (1907, p. 173) gives curves showing that the weight of children fed on sterilised milk is almost identical with that of a normal breast fed baby. This does not show that sterilised milk is a desirable substitute for the breast, since an artificially fed child is always in danger of digestive troubles, but it shows that, granted the necessity for artificial feeding, boiled milk is a suitable substitute. A few dispensaries in France use pasteurised milk for the artificially fed infants, but although I have been unable to obtain any published statistics, I understand that the results have not been superior to those obtained with sterilised milk. Nor are there any weight curves available for comparison of babies fed on raw milk, although a few experiments were made by Bresset who did not find it in any way superior to sterilised milk for normal children.

The question of the modification of the nutrient properties of milk by boiling has often been attacked from the experimental side, but owing to the care required in bringing up young animals upon a foreign milk, boiled or unboiled, and the small number of animals used for the experiments, the results are not so satisfactory as could have been wished.

Bolle (1903) states that guinea-pigs fed on boiled milk got Barlow's disease, whereas those fed on raw milk did well. Guinea-pigs are however not suitable for these experiments, and the results have not been confirmed by Bartenstein (1905). This last observer also fed dogs on boiled and preserved milk (two on each). He does not consider that there was any evidence of ill-health, but there appear to have been complications.

Keller (1904) fed mice on raw, boiled, and sterilised milk, and found that all did equally well; young dogs fed both on fresh or sterilised milk also did equally well, no difference being detected after three months.

Price (1904) found that calves fed on raw milk gained weight, much better than on sterilised milk. Whilst fed upon the latter they had diarrhoea. The experiment was only carried on over 8 days for each kind of milk, and moreover, some calves which did badly on raw milk, subsequently improved on pasteurised milk. Price considers the digestibility to be slightly impaired by boiling.

Peiper and Eichoff (1904) found that dogs became anaemic and the bone rarified after prolonged feeding on slightly sterilised milk.

The experiments of Brüning (1906) are especially noteworthy; he finds that when the milk of a foreign species is used, better results are obtained with boiled than with raw milk, and this as a result of prolonged experiments on pigs (omnivora), dogs (carnivora), and rabbits (10 days experiment), guinea-pigs (14 days experiment) and goats (herbivora).

The experimental evidence, therefore, although not absolutely concordant, certainly seems to show that when the milk of another species is used, there is no marked nutritional difference between raw, boiled, or even sterilised milk, a result which the observations about to be described entirely confirm.

Experiments.

Rats were selected for our experiments. The animals were as nearly as possible a fortnight old at the commencement of the experiment. They were kept in batches of a dozen in airy cages sufficiently large for them to obtain plenty of exercise in climbing about the wires. They were fed twice a day throughout the experiment—morning and evening—on bread and milk, both bread and milk being weighed daily, and adjusted as nearly as possible, so that, although they had plenty to eat, there should be no stale food left in the cages.

The rats in each case were weighed daily all together; in the following figure the results were obtained by dividing the weight by the number of rats.

Series A received bread and milk from the Walker Gordon Laboratories delivered fresh and kept at 0° C. in the cold room until just before use.

Series B received bread and the same milk as Series *A*, but raised to 96° C. just before using.

Series C received bread and sterilised milk. The sterilised milk used was made from the full-cream dried milk of the West Central

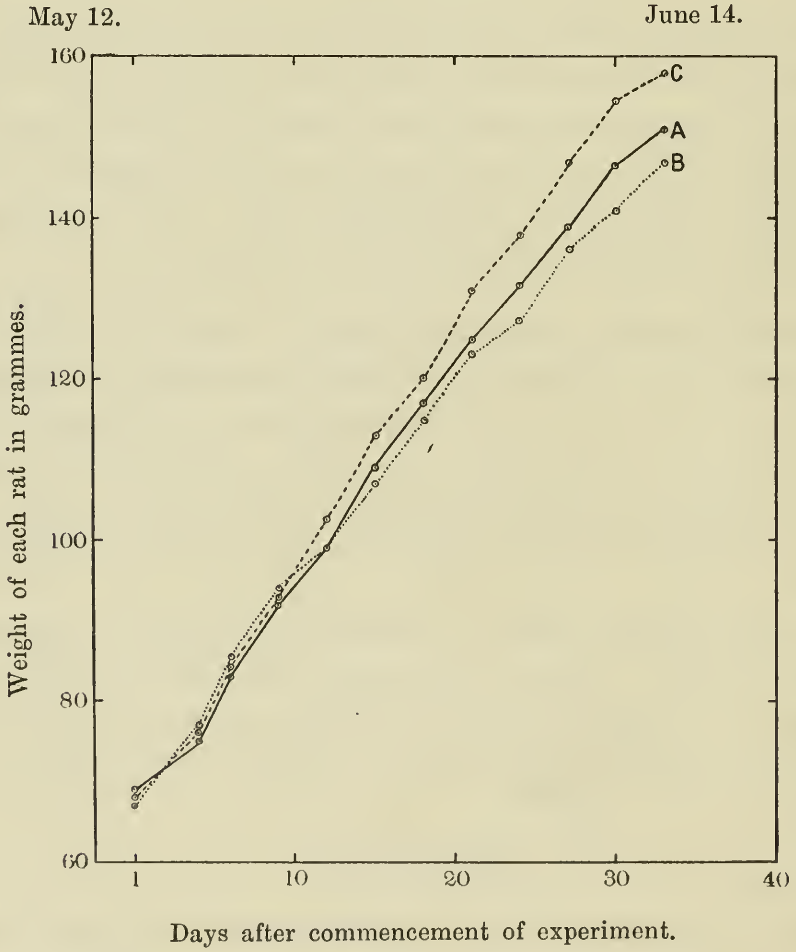

Fig. 1. Showing growth of rats in grammes.

Surrey Dairy, in a 14 % solution, this being almost precisely the average composition of cow's milk.

The details of the results are given in the Table.

Table showing weight of rats used in Series A, B, C.

	Series A (Raw milk)			Series B (Boiled milk)			Series C (Sterilised milk)			Food in % of body weight	
Date	Total wt. in grams	No. of rats	Average wt. per rat	Total wt. in grams	No. of rats	Average wt. per rat	Total wt. in grams	No. of rats	Average wt. per rat	Bread	Milk
May 12	837	12	69·7	800	12	66·6	815	12	67·9	20 %	30 %
,, 15	900	12	75·0	919	12	76·5	912	12	76·0	—	—
,, 18	1002	12	83·5	1027	12	85·5	930	11	84·5	—	—
,, 21	1107	12	92·2	1120	12	93·3	1020	11	92·7	—	—
,. 24	1187	12	98·9	1185	12	98·7	1132	11	102·9	16	24
,, 27	1310	12	109·1	1285	12	107·0	1242	11	112·9	—	—
,, 30	1403	12	116·9	1382	12	115·1	1322	11	120·1	—	—
June 2	1490	12	124·1	1470	12	122·5	1438	11	130·7	13	20
,, 5	1582	12	131·8	1552	12	129·3	1820	11	138·1	—	—
,, 8	1670	12	139·1	1632	12	136·0	1625	11	147·7	11	16
,, 11	1755	12	146·2	1688	12	140·6	1710	11	154·5	—	—
,, 14	1820	12	151·6	1770	12	147·5	1740	11	158·1	10	15
	Increase % = 217·5			Increase % = 221·5			Increase % = 232·8				

Of the three dozen rats, one died in Series *A* a few days after the beginning of the experiment. Of the others it would be difficult to say which batch seemed the healthiest: they all did splendidly: no evidence was obtained of any digestive disturbance; the coats of all were in perfect condition, and the rats exceptionally fine. That they were neither anaemic nor rickety was sufficiently evidenced by their great agility in climbing about the cages, nor was any evidence of such obtained by examination.

The experiments were brought to an end by the detection of an early pregnancy in one of the rats of Series *C*, evidently about the twelfth day; 12 days were therefore knocked off the experiment, so that no complications appear on the curve here given. About a week after the discovery of the first pregnancy, nearly all the females in all three series were found to be pregnant: they went to term and had large families of healthy rats.

In Figure 1 it will be seen that the rats of Series *C* (sterilised milk) gained slightly more weight than the other series. The curve of Series *A* rises higher than *B*, but the initial weight was greater; the actual gain per cent. of *B* was greater than that of *A*, as shown in the table. In another series of experiments also on rats, with raw and boiled milk, the rats fed on raw milk did slightly better as regards weight than the series on boiled milk, but two died of each lot and the rats fed on boiled milk had what appeared to be a nasal catarrh for some weeks during the experiments, which probably contributed to the slight difference in weight: both series were in splendid condition, and all the females had large and healthy families at the age of about eleven weeks.

There would, therefore, appear to be no diminution in nutritive properties for rats, by boiling or even evaporating and drying the milk at 120° C.

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MULTIPLICATION OF BACTERIA AND THE INFLUENCE OF TEMPERATURE AND SOME OTHER CONDITIONS THEREON.

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1 Figure.

THE methods which have been employed for estimating the rate of growth of bacteria are numerous.

Nägeli (1887) endeavoured to estimate the rate of growth by studying the amount of acid produced by an acid-producing organism.

Buchner, Longard and Riedlin (1887) studied the rate of growth of the cholera bacillus, using the plating-out method of Koch. They plated-out the culture at the beginning and at the end of a period of from 2—5 hours. The generation time was calculated from the formulae

$2^n = \frac{b}{a}$ where a = No. of bacteria at beginning,

b = No. of bacteria at end

and n = No. of generations,

and $G = \frac{T}{n}$ where G = generation time and T = time of experiment.

The results obtained for G varied from 19 to 40 minutes at 37° C. This appears to be a large difference, but, as pointed out by Hehewerth (1901), Buchner did not allow for the initial "lag" which most observers find when a fresh culture is made.

Rahn (1900), working chiefly with *B. fluorescens*, has studied the question of this early lag; he found that the minimum generation time at 24° C. occurred 6—21 hours after inoculation. After this the growth was very slow up to about 48 hours.

Klein (1900) counted the number of stained bacteria in a given field, knowing the area of the field in its relation to the area covered by a drop spread out on a film.

Hehewerth (1901) published a number of experiments on the growth of *B. coli* and *B. typhosus*. He worked with large numbers of bacteria, the first estimation generally giving a count of several millions.

He found that there is an initial period after inoculation during which growth is almost absent: the length of time of this "lag" varies with the age of the culture used for inoculation and with the species of the bacillus. With a 19 hours old culture of *B. coli*, the lag at 37° C. lasted rather over one hour: with *B. typhosus* it lasted over two hours.

A culture having once started growing, the numbers run up quickly and then remain fairly constant for some time, subsequently slowly decreasing.

The generation time of *B. coli* at 37° C. in broth he found to be 21—31 minutes, the average being 23 min. 24 sec. and in peptone water to be 28—32 minutes, or sometimes rather more, depending upon the age of the culture. The generation time for *B. typhosus* averaged 33 min. 24 sec. in broth, and 45 min. 37 sec. in peptone water.

Boland (1902) tried to estimate rate of growth by using a standard turbidity.

Müller (1903) used cultures of bacteria isolated by him from frozen material: with slight variations the generation times were the same for the different bacteria at the same temperature, viz., about 50—60 minutes at 30° C. during the first 12 hours, with a lag of nearly two hours; and about 54—70 minutes at 25° C. with a lag of about three hours, or rather more.

He usually started the experiments with a culture containing about 1000—2000 bacteria per c.c.

Barber (1908) worked chiefly with *B. coli*. By means of a fine capillary pipette he removed a single bacterium and determined the actual rate of division. He found no preliminary lag if the bacteria were inoculated into a medium to which they were accustomed. He found that the generation time gradually decreased up to about 40° C., after which it increased.

For *B. coli* he obtained the following times with individual variations of several minutes:

20° C.	60 mins.	40° C.	17 mins. or rather more
25° C.	41 mins.	42° C.	19—20 mins.
30° C.	29·7 mins.	45° C.	30—34 mins.
37° C.	17—21 mins.	50° C.	no growth.

His figure for 37° C. is lower than that of other observers.

The rate of multiplication of bacteria increases from 2—3 times between the temperatures of 20° C. and 30° C. (cp. Hehewerth and Barber).

The optimum temperature differs for different species, and the temperature at which they are kept as stock cultures in the laboratory has been found to have some influence in determining the subsequent rate of growth at any particular temperature.

Methods.

Throughout these experiments the same method has been used, the details being reproduced each time as far as possible.

The cultures used had all been kept at room temperature for over one year. The species of bacteria used were *B. coli, B. typhosus* and *B. enteritidis* Gaertner. A fresh agar culture was made and kept at room temperature for 24 hours and a sub-culture was made from this into about 5 c.c. of broth medium.

The broth culture was then allowed to grow for about 20 hours also at room temperature. With the organisms employed, it was found by experiment that a millionfold dilution of this culture afforded a suitable number of organisms to use for the beginning of an experiment, viz., 200—500 bacteria per c.c.[1]

The culture was kept in the dark at the desired temperature. The temperature varied within half a degree centigrade of that recorded.

At intervals after incubation a definite number of drops (1 drop = ·02 c.c.) were removed by means of a standard capillary pipette and plated.

The actual number of drops required to produce a reliable plate at different periods of incubation had to be discovered by experiment. Two or three plates were made for each estimation.

In the later stages of the experiments the culture required dilution before plating; this was carried out as rapidly as possible by dropping one drop of the culture into the required amount of sterilised water, shaking well, and then with another similar pipette the desired number of drops of the dilution were plated as usual. The whole time required was about two minutes, and control experiments showed that there was no deleterious effect from the distilled water during this short time.

Results of Experiments.

The observations show that there are four phases in the bacterial life of a culture:—(1) an initial period of slow or of no growth; (2) a period of regular growth, the rapidity varying slightly at the same temperature, but differing widely for different temperatures; (3) a period when the numbers remain more or less stationary; (4) a period when the numbers of living bacteria are diminishing.

Period I.

All observers, except Barber (1908), record an initial lag varying in extent at different temperatures. In all my experiments I found a very definite lag, during which the number of bacteria per drop remained almost constant.

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[1] The number of organisms mentioned throughout this paper refers to estimates formed from plating out on agar. This method does not give 100 % of the organisms present but with the same sample of culture medium affords a constant error.

With *B. coli* and *B. enteritidis* Gaertner this latent period was found to extend to from one to six hours as the temperature varied from 42° C. to 20° C. In the case of *B. typhosus* the lag was rather longer at each temperature.

This lag agrees almost exactly with that observed by Hehewerth (1901) who used cultures of very nearly the same age (19 hours). Rahn (1906) states that it is less if the inoculation is fairly heavy.

Period II.

The lag being over the bacteria now enter upon a phase of rapid growth.

The rate of growth has been calculated by various observers on the assumption that the bacteria are all in a state of active and regular division so that the number increases logarithmically. By the method described, I have been able to ascertain that this is the case for a considerable length of time after the culture first starts growing.

The actual figures showing the increase in numbers of *B. coli*, *B. typhosus* and *B. enteritidis* Gaertner, at temperatures 20—42° C. are set out in Tables I, II and III respectively. In parallel columns are placed the average logarithmic differences per hour, the proportional increase in number per hour and the mean generation times as calculated[1] from the observations.

In Fig. 1 the logarithms of the numbers of *B. coli* found in unit volume at different intervals have been plotted against time. This has been done for various temperatures and the points fall upon straight lines. The slope of these lines is different at the various temperatures, the rate of growth being proportional to the tangent of the angles made by them with the abscissa.

Similar graphs are obtained if the figures for *B. typhosus* and *B. enteritidis* Gaertner be also plotted in the same way.

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[1] During the time that growth proceeds logarithmically the number of generations in any interval of time $t_1 - t$ is measured by the power to which 2 must be raised to produce the same increase as the proportion between the number of bacteria at time t_1 and t.

$$2^n = \frac{\text{number at } t_1}{\text{number at } t};$$

taking logarithms the equation can be expressed:

$$n = \frac{\log \text{number } t_1 - \log \text{ number } t}{\log 2};$$

n is the number of generations in time $t_1 - t$ and the generation time is $\frac{t_1 - t}{n}$.

In the experiments in the tables, the ascertained period of "lag" was allowed to elapse before the first observation was made.

For a given volume of culture fluid the time during which the bacteria continue to divide at a maximum rate depended upon the insemination and the temperature, being shorter if the inoculations were heavy and at the higher temperatures.

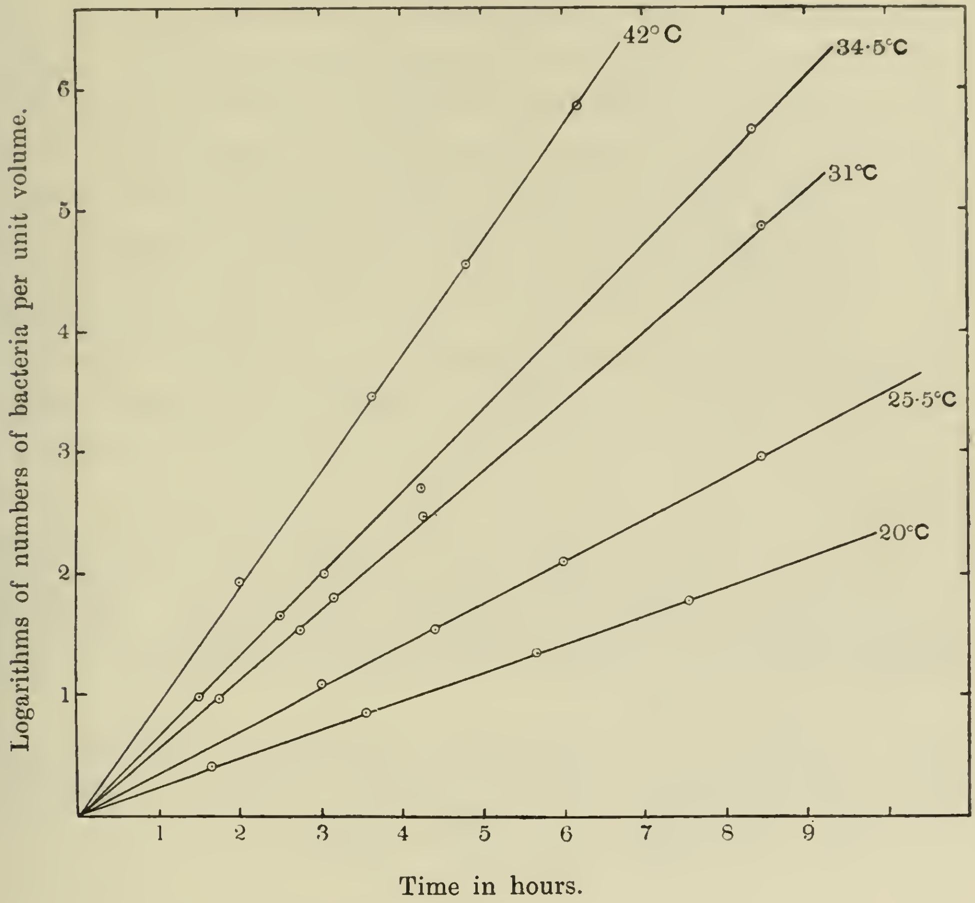

Fig. 1.

The results recorded below are those obtained with an insemination of 200—500 per c.c. Under these circumstances, and discounting the period of lag, the length of time over which *B. coli* and *B. enteritidis* Gaertner maintained the maximum rate was approximately:

7½ hours at 37° C.
8—8½ „ „ 30° C.
12—15 „ „ 25° C.
20—24 „ „ 20° C.

B. typhosus grew more slowly and the logarithmic increase persisted longer at all temperatures.

At 20° and 25° C. the logarithmic increase was maintained almost up to the time when the medium contained a maximum number of living bacilli, but at the higher temperatures the rate fell off considerably before this point was reached.

Periods III and IV.

After the culture has ceased growing logarithmically the rate of growth slackens gradually, but is still fairly active until the number of bacteria reaches several million per drop (·02 c.c.), that is several hundred million per c.c. At this stage the number of living bacteria present appears to remain fairly constant for some time (from two to five days according to the temperature) after which it begins to decrease slowly.

The results obtained with *B. coli* resembled those of *B. enteritidis* Gaertner, except that the maximum number obtained is about double.

There appears therefore to be a maximum number of bacteria which a unit volume of medium is capable of supporting: this means that for that particular organism the conditions are no longer favourable for increased growth. This may be due to the using up of some constituents of the broth or it may be due to some inhibitory substance produced by the organisms themselves in the process of metabolism. I have not carried out any investigations on these points. 400,000,000 to 800,000,000 per c.c. of broth seems to be the maximum for *B. coli* and *B. enteritidis* Gaertner.

After two days at 37° C. and sometimes before, it is difficult to get an accurate estimation as there is a great tendency to agglutinate, especially with *B. coli* and *B. enteritidis* Gaertner.

Influence of temperature upon generation time.

The effect of temperature was studied over the range between 20 and 50° C. Up to 42° the generation time was reduced, but above this it apparently increased, and at 50° diminution replaced increase in the number of organisms.

The experiments of Hehewerth (1901) and Barber (1908) showed that the generation time was reduced one-half to one-third by raising the temperature from 20° to 30° C. This is an effect of the same order as that of temperature upon many common chemical reactions. The logarithms of Barber's values for mean generation times between 20° and 37° C. plotted against temperature fall upon a straight line, showing that each rise of one degree throughout this range produces the same proportionate effect.

My observations for *B. coli* and *B. typhosus* are in accord with those of Barber. The influence of temperature upon the growth of *B. enteritidis* Gaertner I find to be decidedly less. The temperature effect upon the growth of these three organisms is fairly constant between 20° and 35° C. Each rise of one degree produces the same proportionate increase in the rate of growth. The logarithms of the mean generation times (20°—35°) from Tables I, II and III plotted against temperature show a linear relationship, signifying that the effect of temperature upon the growth of these bacteria accords with the Arrhenius-Van 't Hoff law within the error of the experiments.

The increase in rate of growth per 10° rise in temperature obtained from the drawn lines is 2·2 for *B. coli* and *B. typhosus* and 1·7 for *B. enteritidis* Gaertner.

Above 35° C. the effect of temperature in diminishing the mean generation time is, as found by Barber, distinctly less.

Summary.

The species of organisms used were *B. coli*, *B. typhosus* and *B. enteritidis* Gaertner. With these organisms:

(1) When a fresh broth culture is made with a small inoculation there is a period during which there is no increase in the number of bacteria present.

(2) When this period is over the bacteria commence to divide regularly; this is shown by the fact that the logarithms of the numbers plotted against time are found to fall on a straight line. This regular growth persists until (or nearly until) a maximum has been reached, after which the numbers remain more or less constant and then slowly decline.

The time necessary for a complete division to take place (generation time) was determined for various temperatures between 20° C. and 42° C.

(3) The effect of temperatures between 20° and 35·3° C. upon the rate of multiplication is in accordance with the Arrhenius-Van 't Hoff law; above this temperature the effect diminishes.

I have much pleasure in thanking Dr C. J. Martin for his invaluable assistance and interest throughout this research and also Miss Müllenbach for kindly preparing the diagram.

TABLE I.

Growth of B. coli at temperatures 20° *to* 42° C.

Temp.	Time after commencement of experiment hrs.	mins.	No. of bacteria per drop	Logarithm of number per drop	Average Log. difference per hour = velocity constant	Proportional increase in numbers per hour	Mean generation time in hours
42° C.	2	0	87	1·94	·94	8·7	·32
	3	40	2,876	3·46			
	4	50	36,675	4·56			
	6	10	739,200	5·87			
34·3° C.	1	30	9	·97	·68	4·8	·44
	2	30	43	1·63			
	3	5	105	2·02			
	4	15	499	2·70			
	8	22	476,666	5·68			
31° C.	1	45	10	1·00	·57	3·7	·53
	2	45	34	1·53			
	3	10	65	1·81			
	4	22	288	2·46			
	8	27	72,533	4·86			
25·5° C.	3	0	12	1·08	·35	2·2	·86
	4	25	33	1·52			
	6	0	128	2·11			
	8	27	893	2·95			
20° C.	12	15	243	2·38	·23	1·7	1·30
	14	10	720	2·85			
	16	15	2,166	3·33			
	18	10	5,066	3·70			
	22	30	46,666	4·67			

TABLE II.

Growth of B. typhosus at temperatures 20°—34·3° C.

Temp.	hrs.	mins.	No. of bacteria per drop	Logarithm of number per drop	Average Log. difference per hour = velocity constant	Proportional increase in numbers per hour	Mean generation time in hours
34·3° C.	1	30	7	·84	·55	3·5	·55
	2	30	24	1·38			
	4	0	152	2·18			
	9	20	195,000	5·29			
	26	20	4,133,000	6·62			
31° C.	1	50	7	·84	·40	2·5	·75
	2	45	17	1·23			
	4	30	82	1·91			
	9	25	13,600	4·13			
	26	25	3,966,000	6·98			
25° C.	3	50	10	1·0	·23	1·7	1·30
	6	10	36	1·56			
	9	35	178	2·25			
	26	30	3.566,000	6·55			
20° C.	6	15	10	1·0	·18	1·5	1·67
	8	37	28	1·45			
	9	38	36	1·56			
	26	35	51,145	4·71			
	54	10	4,933,000	6·69			

TABLE III.

Growth of B. enteritidis Gaertner at temperatures 20° *to* 42° C.

Temp.	Time after commencement of experiment		No. of bacteria per drop	Logarithm of number per drop	Average Log. difference per hour = velocity constant	Proportional increase in numbers per hour	Mean generation time in hours
	hrs.	mins.					
42° C.	2	20	83	1·92	·81	6·5	·37
	4	0	1,940	3·28			
	5	0	13,417	4·12			
	6	20	176,400	5·24			
34·3° C.	1	30	13	1·12	·59	3·9	·51
	2	30	43	1·63			
	3	0	80	1·90			
	3	30	179	2·25			
	6	15	8,960	3·95			
	12	25	3,580,000	6·55			
	25	30	5,366,000	6·72			
	49	40	10,833,000	7·03			
	121	0	6,266,000	6·79			
31° C.	1	45	13	1·11	·54	3·5	·56
	2	45	44	1·64			
	3	15	85	1·92			
	6	35	4,050	3·60			
	8	15	44,000	4·64			
	12	30	1,480,000	6·17			
	25	30	9,000,000	6·95			
	49	40	8,400,000	6·92			
	121	0	4,600,000	6·66			
26° C.	4	0	25	1·40	·42	2·6	·71
	4	45	57	1·75			
	5	30	112	2·04			
	6	15	208	2·31			
	6	45	361	2·55			
	12	0	56,933	4·75			
	25	30	9,125,000	6·96			
	49	40	9,600,000	6·98			
	121	0	10,466,000	7·01			
20° C.	6	40	28	1·44	·29	2·0	1·0
	7	45	56	1·75			
	8	35	98	1·99			
	12	45	1,546	3·18			
	26	0	7,581,400	6·87			
	49	40	10,433,000	7·01			
	121	0	10,066,000	7·00			

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VI.

Die Synthese der Fette im Tierkörper.

Von

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Das physiologische Interesse an dem Fettstoffwechsel betrifft hauptsächlich die Fettsäuren, denn diese letzteren Bestandteile der Fette sind es, die deren grossen Wert als Energieerzeuger bestimmen. Die chemischen Probleme über den Ursprung der langen Ketten von nichtoxydierten Kohlenstoffatomen einerseits und der vollständigen Oxydation dieser spröden Verbindungen andererseits gehören zu den schwierigsten und dunkelsten in dem ganzen Gebiete der Physiologie. Diese Fettsäuren sind in vielen, dem Tier-

örper eigenen Verbindungen, anwesend, wie z. B. in den einfachen Glyze-
den, die in mannigfaltigen Formen vorhanden sind, sowie in den mehr
omplizierten Lipoiden, deren Anzahl und Zusammensetzung bisher nur
enig bekannt ist. Während es uns wohl bekannt ist, dass die einfachen
lyzeride mit der Nahrung in den Körper eingeführt, im Bindegewebe in
er gleichen Form, in welcher sie in der Nahrung vorkommen, abgesetzt
erden, wissen wir auch, dass diese Glyzeride jedenfalls auch aus Kohle-
ydraten, vielleicht aber sogar aus einfacheren, anderen Quellen entsprin-
enden Verbindungen synthetisiert werden. Was die komplizierteren Lipoide
nbetrifft, so finden sie sich allerdings in nennenswerten Mengen in der von
ns eingenommenen Nahrung vor, doch ist es höchst unwahrscheinlich,
ass der für unsere Gewebe notwendige Bedarf von der bisher noch nicht
ewiesenen und vorderhand unwahrscheinlich erscheinenden Möglichkeit ab-
ängig sei, dass solche unbeständigen Substanzen unverändert aus dem Ver-
lauungskanal in die Zellen gelangen. Es liegt somit genügend Ursache vor
u der Annahme, dass während die Fettsäuren selbst entweder zugleich mit
ler Nahrung eingeführt oder aber in dem tierischen Körper synthetisch her-
gestellt werden können, die komplizierteren Verbindungen, in denen diese
ettsäuren auftreten, immer von der synthetischen Wirksamkeit der Zellen
bhängig sind.

Die physiologische Synthese der Fette lässt sich somit in die folgenden
Abschnitte zerlegen:

I. Die Synthese der höheren Fettsäuren.
II. Die Synthese der einfachen Glyzeride dieser Fettsäuren.
III. Die Synthese anderer Verbindungen dieser Säuren, die unter der Bezeichnung „Lipoide“ zusammengefasst werden.

I. Die Synthese höherer Fettsäuren wird nicht bloss durch den tierischen, sondern auch durch den Pflanzenorganismus ausgeführt. In letzterem Falle ist es wahrscheinlich, dass das zu der Synthese angewandte Material aus den Kohlehydraten der Pflanzengewebe stammt, obgleich dies noch nicht ganz sicher bewiesen ist. Pflanzenöle finden sich meistens in Samen und Nüssen vor, und der Umstand, dass unreife Samen und Nüsse hauptsächlich Kohlehydrate, die reifen aber mehr Öle enthalten, wird gewöhnlich dadurch erklärt, dass das Öl während des Reifeprozesses innerhalb des Samens oder der Nuss aus den in denselben vorhandenen Kohlehydraten gebildet wird. Es ist jedoch durchaus nicht bewiesen, dass der Vorgang in obiger Weise stattfindet und es ist möglich, dass das Öl an anderer Stelle gebildet und von aussenher dem reifenden Samen zugeführt wird. Der Transport von Fettsäuren ist nie nachgewiesen worden; ziehen wir aber die Schwierigkeit in Betracht, die dem Nachweis der Abgabe von Fett aus den Bindegeweben an die Organe innerhalb des Tierkörpers anhaftet.

so ist es durchaus nicht unmöglich, anzunehmen, dass der Transport höherer Fettsäuren innerhalb der Pflanzen sich der Beobachtung entziehen mag.

Obwohl es somit wahrscheinlich ist, dass die Pflanzenöle aus Kohlehydraten gebildet werden, so ist es doch nicht angängig, diese Behauptung als bewiesen zu betrachten. Die wohlbekannten durch Lawes und Gilbert in Rothamstead an Tieren ausgeführten Versuche stellten schon vor 60 Jahren die Möglichkeit der Bildung von Fett aus Kohlehydraten fest. So wichtig jedoch die Feststellung dieser Tatsache war, so blieb es doch anderen vorbehalten, die zwischenliegenden Stufen des oben erwähnten Vorganges folgerichtig auszuarbeiten. Das gründliche Verständnis dieser Vorgänge ist zu einem klaren Verständnis der Tierchemie durchaus notwendig und muss erreicht werden, bevor wir hoffen dürfen, die während Krankheiten in ihrem Laufe gestörten Lebensprozesse zu überwachen und zu regulieren. Die beste bisher gelieferte Erklärung stammt von Hoppe-Seyler und Nencki und beruht darauf, dass Zucker sich leicht in Milchsäure umsetzt und Milchsäure, den anderen α-Oxysäuren ähnlich, wie nunmehr bekannt ist, Ameisensäure oder Kohlenoxyd und Wasser abgibt, und zur Entstehung des nächsten Aldehyds in der Reihe der aliphatischen Homologen Anlass gibt, in diesem Falle des Acetaldehyds. Vermittelst Kondensation könnte aus Acetaldehyd eine Reihe von Verbindungen entstehen, die eine gerade Zahl von Kohlenstoffatomen enthalten und darin den im natürlichen Zustande in Pflanzen, sowohl als in Tieren vorkommenden Fettsäuren ähnlich sind.

Die darauf bezüglichen Untersuchungen zerfallen in 3 Abschnitte:

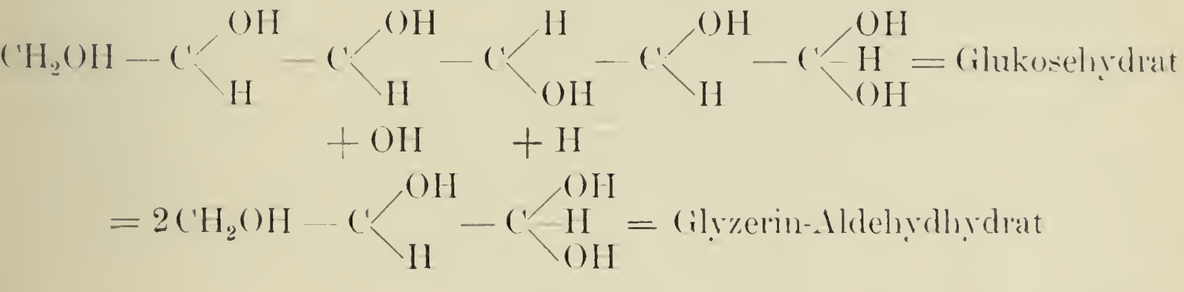
1. Die Bildung von Milchsäure oder von einer naheverwandten, drei Kohlenstoffatome enthaltenden Verbindung aus Kohlehydraten.
2. Die Bildung einer weitern, der Kondensation fähigen und zwei Kohlenstoffatome enthaltenden Verbindung aus der vorhergehenden Verbindung.
3. Die Kondensation und Bildung der gradzahligen Glieder der Reihe von aliphatischen Monokarbonsäuren.

Die Bildung von Milchsäure oder verwandter Verbindungen mit 3 Kohlenstoffatomen aus Zucker.

Die wohlbekannte Zersetzung der Zucker vermittelst Alkalien, welche die Grundlage der Mooreschen Reaktion bildet, wurde von Nencki untersucht. Als das wichtigste Produkt dieser Zersetzung erscheint die Milchsäure. Zucker, welche unveränderte Aldehyd- oder Ketongruppen enthalten, geben sämtlich die Mooresche Reaktion und werden teilweise in Milchsäure umgesetzt. Ausser Glukose verhalten sich auch Fruktose, Galaktose, Mannose, Laktose, Maltose, und die Pentosen, Arabinose und Xylose in derselben Weise. Rohrzucker dagegen reagiert nicht in dieser Weise, bis vermittelst der Inversion lie gebundenen Aldehyd- und Ketongruppen freigemacht werden. In einer Lösung, die 10% Glukose und 20% Kalilauge enthält und während 24 Stunden Körpertemperatur ausgesetzt wird, entsteht Milchsäure in einer Menge, die 0% des in der Lösung enthaltenen Zuckers entspricht. Selbst bei niedrigeren Temperaturen, besonders unter der Einwirkung des Sonnenlichtes, werden nit Hilfe der anorganischen Basen grosse Mengen von Milchsäure aus Zucker ebildet. Es ist, mit Rücksicht auf die Tierchemie von Interesse, dass, nach Nencki, gewisse organische Basen, Neurin z. B., das gleiche Resultat hervorzubringen imstande sind, obgleich Ammoniak und die alkalischen Karbonate in dieser Beziehung wirkungslos bleiben.

Es ist natürlich wohlbekannt, dass unter den vielen durch Mikroorganismen in Zuckerlösungen verursachten Umwandlungen die Bildung von Milchsäure die gewöhnlichste Erscheinung ist. Die Mikroorganismen, die hauptsächlich das Sauerwerden der Milch verursachen, sind, einer Beobachtung gemäss, imstande, gegen 80% Milchsäure aus dem Zucker zu bilden; dieser Umstand scheint darauf hinzuweisen, dass selbst wenn kein einziges der Zuckermoleküle einer teilweisen Umwandlung in Milchsäure entgeht, doch mindestens 60% derselben sich jedes in zwei Moleküle von Milchsäure umgesetzt haben müssen.

Wenn die Behauptung, dass eine Spaltung der Kette von sechs C-Atomen n zwei gleiche Hälften der Bildung von Milchsäure aus Zucker vorausgeht, nd dass jede der beiden Hälften imstande ist, sich in Milchsäure umzuwandeln, der Tatsache entspricht, so lässt sich dieser Vorgang am leichtesten urch Hinzutreten von Wasser in der Mitte des Moleküls erklären, etwa so:

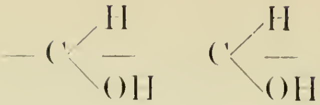


$$CH_2OH - C\begin{smallmatrix}OH\\H\end{smallmatrix} - C\begin{smallmatrix}OH\\H\end{smallmatrix} - C\begin{smallmatrix}H\\OH\end{smallmatrix} - C\begin{smallmatrix}OH\\H\end{smallmatrix} - C\begin{smallmatrix}OH\\H\\OH\end{smallmatrix} = \text{Glukosehydrat}$$

$$+ OH \qquad + H$$

$$= 2CH_2OH - C\begin{smallmatrix}OH\\H\end{smallmatrix} - C\begin{smallmatrix}OH\\H\\OH\end{smallmatrix} = \text{Glyzerin-Aldehydhydrat}$$

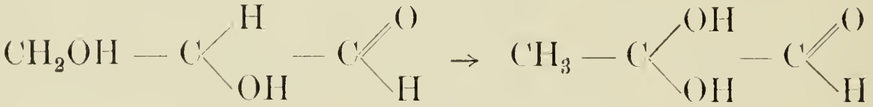
Gleicherweise lässt sich vielleicht der Umstand, dass es nicht möglich t, eine Gärung von Galaktose durch Hefe zu verursachen, während bei lukose, Fruktose und Mannose dieses mit gleicher Leichtigkeit geschieht, der gleichen Weise erklären, falls wir geneigt sind mit Buchner anzuehmen, dass eine Spaltung der Kette von sechs C-Atomen in zwei gleiche Iälften der Bildung von Alkohol und Kohlensäure vorausgeht. Denn in len drei Hexosen, die leicht durch Hefe in Gärung versetzt werden, gruppieren sich die in der Mitte befindlichen Kohlenstoffatome in gleicher Weise bezug auf Hydroxylgruppen und Wasserstoffatome, während bei Galaktose ie Gruppierung sich folgendermassen stellt:



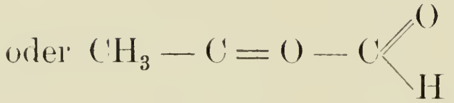
$$-\mathrm{C}\begin{array}{l}\diagup\mathrm{H}\\ -\\ \diagdown\mathrm{OH}\end{array}\qquad \mathrm{C}\begin{array}{l}\diagup\mathrm{H}\\ -\\ \diagdown\mathrm{OH}\end{array}$$

und somit sich nicht sogut zu einer hydrolytischen Spaltung eignet, da der Wasserstoff und das Hydroxyl des eintretenden Wassers von verschiedenen Seiten her in das Molekül eindringen müssten.

Falls Glyzerinaldehyd als erstes Spaltungsprodukt des Zuckers erscheint (und der Umstand, dass ersterer unter genau denselben Bedingungen als Zucker sich in Milchsäure umsetzt, macht dies wahrscheinlich), muss die weitere Umwandlung in Milchsäure durch eine Überführung des Hydroxyls der ersten Alkoholgruppe zu dem andern Ende des Moleküls bedingt werden, so dass es mit dem Wasserstoffatom der Aldehydgruppe den Platz wechselt. Findet die Überführung in zwei Stufen statt, so stellt sich die erste wohl so:



$$\mathrm{CH_2OH}-\mathrm{C}\begin{array}{l}\diagup\mathrm{H}\\ \diagdown\mathrm{OH}\end{array}-\mathrm{C}\begin{array}{l}\diagup\!\!\diagup\mathrm{O}\\ \diagdown\mathrm{H}\end{array}\rightarrow \mathrm{CH_3}-\mathrm{C}\begin{array}{l}\diagup\mathrm{OH}\\ \diagdown\mathrm{OH}\end{array}-\mathrm{C}\begin{array}{l}\diagup\!\!\diagup\mathrm{O}\\ \diagdown\mathrm{H}\end{array}$$



$$\text{oder }\mathrm{CH_3}-\mathrm{C}=\mathrm{O}-\mathrm{C}\begin{array}{l}\diagup\!\!\diagup\mathrm{O}\\ \diagdown\mathrm{H}\end{array}$$

Brenztraubenaldehyd.

Nef (1) hat bewiesen, dass bei der Behandlung von Zucker mit Alkali Brenztraubenaldehyd gebildet wird und sich als Übergangsstufe zur Bildung von Milchsäure darbietet. Bei der Spaltung der Hexosenketten erscheinen somit Glyzerinaldehyd, Brenztraubenaldehyd und Milchsäure und jede dieser Verbindungen könnte vermittelst weiterer Veränderungen die Entstehung von Produkten verursachen, die zu der Kondensation geeignet sind, welche zur Bildung der langen in Fetten enthaltenen Fettsäureketten notwendig ist.

Milchsäure findet sich auch manchmal in beträchtlichen Mengen innerhalb des Tierkörpers vor. So z. B. schieden Gänse nach Entfernung der Leber grosse Mengen von Milchsäure mit dem Harne aus in der Form des Ammoniaksalzes, und da dieses Ammoniak den meisten innerhalb des Körpers aus Eiweiss freiwerdenden Stickstoff enthält, und die Menge der ausgeschiedenen Milchsäure sowohl als des Ammoniaks, mit der Menge des in der Nahrung enthaltenen Eiweisses in Verbindung steht, so ist behauptet worden, dass der Ursprung der Milchsäure mehr im Eiweiss als in einem Kohlehydrat zu suchen ist. Diese Annahme ist jedoch kaum gerechtfertigt. Wenn im Laufe des Eiweissstoffwechsels Ammoniak freigesetzt wird, wird sich dieses letztere mit der ersten und am häufigsten vorhandenen Säure verbinden und durchaus nicht notwendigerweise allein mit solchen, die aus Eiweiss stammen. Der Umstand, dass sich ein Milchsäurekern in der Harnsäure findet und dass bei den betreffenden Tieren Harnsäure durch Ammoniumlaktat ersetzt wird, beweist durchaus nicht, dass all die Milchsäure, welche von ihnen ausgeschieden wird, sich normalerweise in Harnsäure umsetzen würde. Keinesfalls wird mehr als ein Viertel der vorhandenen Menge sich auf obenerwähnte Weise umsetzen, schon weil jedes Milchsäuremolekül vier Ammoniakmoleküle braucht, um in Harnsäure umgesetzt zu werden, — es braucht jedoch nur eins, um Ammoniaklaktat zu bilden.

Mehrere andere, zur Begründung der Theorie, dass Milchsäure im Laufe der Eiweisszersetzung im Tierkörper gebildet wird, dienende Beweise klingen ebensowenig überzeugend. Die Versuche, die von Asher und Jackson am Blut von Hunden, deren Eingeweide entfernt worden waren, gemacht wurden, stellten eine Ansammlung von Milchsäure im Blute fest, begleitet von anderen stickstoffhaltigen Substanzen, die in Alkohol löslich waren, und wurden im obigen Sinne erklärt. Aus der Steigerung der in Alkohol löslichen, im Blute vorhandenen Stickstoffmenge berechneten sie die Menge des zersetzten Eiweisses, die in sechs Versuchen gleich 55 bis 106 mg war; die Quantität der gleichzeitig vorgefundenen Milchsäure war 43 bis 106 mg oder, mit anderen Worten, 90% des Eiweisses, was als eine unwahrscheinlich grosse Menge erscheint. Es ist allerdings möglich, dass ein Teil der Stickstoffderivate des Eiweisses in Alkohol unlöslich war, doch falls dieses der Fall gewesen, enthielten sie wohl viel Kohlenstoff und jedenfalls kann eine quantitative Bestimmung der Milchsäure im Blute kaum ausgeführt werden, denn der unvermeidliche Verlust soll ungefähr 50% betragen (2). Somit scheint es also unwahrscheinlich, dass die Menge der im Blute vorgefundenen Milchsäure klein genug war, um aus dem Eiweiss herzustammen. Es ist wahr, dass Milchsäure auch durch Hydrolyse der Amidogruppe aus Alanin gebildet werden kann, dass Alanin, obgleich es nicht an und für sich in beträchtlichen Mengen im Eiweissmolekül vorhanden, doch in manchen der komplizierteren Aminosäuren anwesend ist, die ihrerseits Bestandteile des Eiweisses bilden, so dass eine gewisse Menge Milchsäure wahrscheinlich im Laufe des Eiweissstoffwechsels gebildet wird. Die verschiedenen Hypothesen, welche mit Bezug auf die Entstehung der in den Muskeln enthaltenen Milchsäure aufgestellt worden sind, haben jedoch deren Ursprung unerklärt gelassen. Fletcher und Hopkins (3) haben bewiesen, wie unzuverlässig die bisherigen Bestimmungen der Milchsäure in Muskeln sind. Obwohl es ihnen gelungen ist, vieles bisher auf dem Gebiete der Entstehung der Milchsäure Rätselhafte zu erklären, so haben sie sich doch nicht damit befasst, die Vorstufe, aus denen diese Säure gebildet wird, zu untersuchen. In der von Magnus-Levy (4) gelieferten Arbeit über die Bildung von Milchsäure und anderen Säuren in der Leber ist keineswegs der Beweis vorhanden, dass die Milchsäure aus Kohlehydraten sich herleiten lässt, obgleich der Verfasser sich dieser Annahme zuneigt. Wenn auch das Vorkommen von Milchsäure im Tierkörper das Verständnis der Bildung von Fettsäuren aus Kohlehydrat erleichtern würde, so fehlt doch der sichere Beweis, dass diese Milchsäure aus Kohlehydrat wirklich gebildet wird, wie wahrscheinlich dieses auch sein mag.

2. Die Bildung einer zwei Kohlenstoffatome enthaltenden, kondensationsfähigen Verbindung.

Wie bereits oben erwähnt, stellte Nencki die Hypothese auf, dass die höheren Fettsäuren auf folgende Weise sich bilden: aus Zucker entsteht Milchsäure, aus dieser wieder Acetaldehyd und Ameisensäure und vermittelst Kondensation des ersteren sollten Produkte erhalten werden, die sich in Fettsäuren umsetzen liessen. Die Zersetzung von Milchsäure unter Bildung von Acetaldehyd kommt unter vielen Bedingungen vor: bei Erhitzen der Säure selbst auf eine hohe Temperatur, oder bei Erhitzen mit verdünnter Schwefelsäure auf 130°, bei Behandlung mit Schwefelsäure und Mangandioxyd oder Bleiperoxyd, und auch bei Elektrolyse. Nach Dakin (5) findet dieselbe Reaktion statt, wenn Alanin mit Wasserstoffsuperoxyd und Spuren von Ferrosulfat behandelt wird: Kohlensäure, Ammoniak und teilweise zu Essigsäure oxydierter Acetaldehyd erscheinen als Resultat dieser Reaktion.

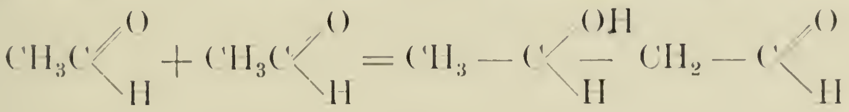
Andere Amidosäuren verhalten sich in gleicher Weise und im allgemeinen werden α-Oxysäuren, wenn auf 200° erhitzt, in niedrigere Aldehyde und Ameisensäure oder Kohlenoxyd und Wasser zersetzt.

Eine ähnliche Zersetzung findet bei Einwirkung von Kalilauge auf Zucker im Sonnenlicht statt; denn unter diesen Bedingungen fand Duclaux (6), dass die Zersetzung des Zuckers zu Milchsäure von der Bildung einer gewissen Menge von Alkohol gefolgt wird, welche letztere Behauptung von Buchner zur Unterstüzung seiner Theorie angeführt wird, dass die alkoholische Gärung sich über Milchsäurebildung vollzieht.

Die Kondensation.

Pasteurs Beobachtung, dass ein Organismus, der Buttersäuregärung in Zuckerlösung verursacht, auch, wenn auf Calciumlaktat übergeführt, Buttersäure produzierte, liess voraussetzen, dass Milchsäure sich in direkter Linie zwischen Zucker und Buttersäure befindet und dass der Bildung der 4-Kohlenstoffkette der Buttersäure eine Synthese zugrunde liegt, nicht aber eine Abspaltung einer fertigen viergliedrigen Kette von dem Hexosemolekül. Diese Synthese wurde von einer Freimachung von Wasserstoff und Kohlensäure, den Bestandteilen der Ameisensäure, begleitet, und somit kann sie als Kondensation von von 2 Acetaldehydmolekülen angesehen werden, zumal wir gesehen haben, dass diese beiden Substanzen unter mehrfachen Bedingungen aus Milchsäure entstehen. Die Bildung einer Normalfettsäure mit einer Kette von vier Kohlenstoffatomen liefert den ersten Schritt zu einer Herstellung von Normalsäuren mit anderen, graden Zahlen von Kohlenstoffatomen und wir müssen uns gleichzeitig vergegenwärtigen, dass während der Buttersäuregärung ausser der Buttersäure noch Kapronsäure gebildet wird, welch letztere zu Handelszwecken aus Zucker in dieser Weise dargestellt wird. Der wichtige Beweis, dass ausser Kapronsäure noch Normalkaprylsäure in kleinen Mengen in den Produkten der Buttersäuregärung des Zuckers anwesend ist, wurde letzthin von Raper (7) geliefert, der somit bewiesen hat, dass Nencki und andere, welche obige Reaktion als den Weg bezeichnet haben, auf dem die höheren Fettsäuren aufgebaut werden, Recht gehabt haben. Die biologische Reaktion, vermöge welcher die Säuren mit 4, 6 und 8 Kohlenstoffatomen (Buttersäure, Kapron- und Kaprylsäure) gebildet werden, würde, weiter fortgesetzt Kaprin-, Laurin-, Myristin-, Palmitin-, Stearin- und Arachinsäure ergeben, welche sämtlich in grösseren oder kleineren Mengen im Milchfett der Säugetiere nachgewiesen worden sind.

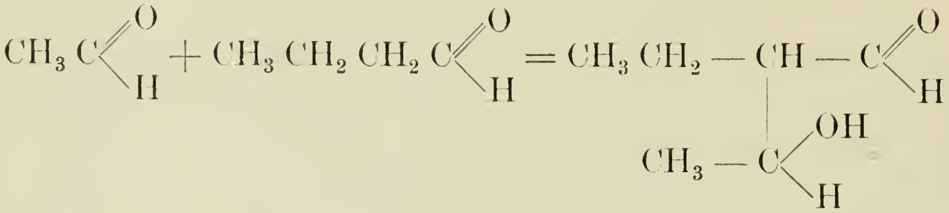
Was den Weg dieser Synthese anbetrifft, so besteht der erste zur Bildung einer 4-Kohlenstoffatomkette führende Schritt in der Kondensation des Acetaldehyds zu Aldol, eine Reaktion, zu der er so leicht geneigt ist.



$$CH_3C\left\langle\begin{matrix}O\\H\end{matrix}\right. + CH_3C\left\langle\begin{matrix}O\\H\end{matrix}\right. = CH_3 - C\left\langle\begin{matrix}OH\\H\end{matrix}\right. - CH_2 - C\left\langle\begin{matrix}O\\H\end{matrix}\right.$$

Aus Versuchen von E. Friedmann (11a) wissen wir, dass sich eine Synthese in der überlebenden Leber vollzieht, wodurch Acetaldehydammoniak in Acetessigsäure und Aceton übergeführt wird; dass es sich hier in erster Reihe um die Bildung von Aldol handelt, deutet die Tatsache an, dass auch der Zusatz von diesem zu der Durchblutungsflüssigkeit zu einer Neubildung von Acetessigsäure führt.

Das Aldol, welches β-Oxybutteraldehyd ist, kann durch Versetzung des β-Hydroxyls und des Aldehydwasserstoffes in Normalbuttersäure verwandelt werden; dieser Vorgang kann durch abwechselnde Oxydation und Reduktion vermittelst Silbernitrat und Jodwasserstoffsäure in vitro leicht ausgeführt werden. Es bestehen jedoch noch andere Kondensationsprodukte des Aldehyds, an deren Bildung mehr als zwei Moleküle teilnehmen, mit dem Resultat, dass längere Ketten aufgebaut werden. Kekulé und Riban haben dergleichen Verbindungen mit sechs Kohlenstoffatomen beschrieben, und da die Aldolkondensation eine Reaktion bildet, die allen Aldehyden eigen ist, so dürfen wir annehmen, dass sämtliche entstehende aldolartigen Körper sich mit Acetaldehyd oder miteinander zu kondensieren und noch längere Ketten zu bilden imstande sind. Somit ist die Hypothese, dass eine solche Kondensation durch Vermittelung biologischer Kondensationsagenzien oder von Enzymen, die Entstehung der in der Natur vorkommenden gradzahligen Glieder der Fettsäurereihe hauptsächlich Palmitin- und Stearinsäure verursacht, ganz annehmbar. Eine Schwierigkeit in der Annahme dieser Hypothese liegt darin, dass wenn ein Aldehyd sich mit einem anderen mehr als zwei Kohlenstoffatome enthaltendem kondensiert, die Aldehydgruppe sich nicht mit der Methylgruppe am Ende des Moleküls, sondern mit der α-Methylengruppe verbindet und die Kette somit keine normale, sondern eine verzweigte wird.



$$\mathrm{CH_3C\overset{O}{\underset{H}{<}} + CH_3\,CH_2\,CH_2\,C\overset{O}{\underset{H}{<}} = CH_3\,CH_2 - \underset{\underset{CH_3 - C\overset{OH}{\underset{H}{<}}}{|}}{CH} - C\overset{O}{\underset{H}{<}}}$$

Um bei derartigen Reaktionen die normalen Ketten der natürlichen Fettsäuren zu erhalten ist es natürlich notwendig, dass die Kondensation immer an den Molekülenden stattfindet.

Nun ist von Raper (8) bewiesen worden, dass bei Kondensation von Aldol mit Aldol die Reaktion in eben solcher Weise vor sich geht, da die Anwesenheit der β-Hydroxylgruppe anscheinend die Kondensation mit dem α-Kohlenstoffatom verhindert. Er kondensierte Aldol mit Aldol durch Kaliumkarbonat bei niedriger Temperatur, oxydierte den Aldehyd durch Silbernitrat, um die entsprechende Säure zu bilden und reduzierte die Hydroxylgruppen vermittelst Jodwasserstoffsäure. Die auf diese Weise erhaltene Säure erwies sich als Normalkaprylsäure.

Die Bildung von Normalketten wird also in dieser Weise stattfinden, solange nur die Kondensation vor der Reduktion der von der vorhergehenden Kondensation übrigen Hydroxylgruppe stattfindet.

Es existieren somit ziemlich viele indirekte Hinweise, die auf die Richtigkeit der Hypothese schliessen lassen, dass die Synthese höherer Fettsäuren aus Kohlehydraten vermittelst einer Kondensation der zwei Kohlenstoffatome enthaltenden Gruppen, wie z. B. Acetaldehyd, ausgeführt wird.

Der Umstand, dass Acetaldehyd eine giftige Substanz ist, und als solche nicht im lebenden Körper geduldet würde, erscheint keineswegs als Hindernis zur Annahme der Hypothese. Denn die Kondensation selbst liefert die Mittel sich der schädlichen Substanz sofort zu entledigen genau so, wie es bei der Synthese der Kohlehydrate in Pflanzen der Fall ist. In den letzteren wird der noch giftigere Formaldehyd zu diesem Zweck verwendet, obgleich derselbe, wenn nicht vollständig vertrieben, die Katalase angreift, welche der Pflanze nötig ist, um die toxische Wirkung des Wasserstoffperoxyds auf das Chlorophyll zu verhindern (9).

Die Beantwortung der Frage, wie die Kohlehydrate in Fett übergeführt werden, beruht somit noch auf einer Hypothese. Ebenso ist es noch unbekannt, an welcher Stelle diese Umwandlung stattfindet. Der Umstand, dass Reduktionsprozesse notwendigerweise daran teilnehmen müssen, lässt vermuten, dass die Leber an diesen Vorgängen beteiligt ist. Versuche sind angestellt worden nach demselben Prinzip, nach welchem Bernard die Bildung von Zucker in der Leber aus Glykogen nachgewiesen hat. In einer angen Reihe von darauf bezüglichen Versuchen, die von mir ausgeführt vurden, fand sich bei ungefähr der Hälfte der Experimente, dass die Menge öherer Fettsäuren, die in Petroleum löslich und in Wasser unlöslich, in verchiedenen Verbindungen in der Leber anwesend sind, grösser wurde, wenn in Teil des feinzerriebenen Gewebes 24—72 Stunden lang bei Körpertemeratur entweder in der Anwesenheit von antiseptischen Mitteln, oder aber teril, erhalten wurde (10).

Als Massstab der Vermehrung dienten zwei Kontrollversuche, in welchen ler Gehalt an Fettsäuren in demselben Material sofort nach dessen Zuereitung bestimmt wurde. Die bei diesen zwei Kontrollversuchen erhalenen Resultate dienten auch dazu, die wahrscheinlichen Grenzen der anaytischen Präzision, nach den angewandten Methoden rund um 1%, festzustellen. Wenn die Steigerung 10% bis 35% betrug, wurde das Resultat ls positiv betrachtet; in gewissen Fällen, wo der Fettgehalt der Leber besonders hoch war, wurde auch eine niedrigere Steigerung, z. B. 5%, als positives Resultat betrachtet, da in diesem Falle die absolute Gewichtszunahme der Fettsäuren an und für sich bedeutend war, z. B. 0,5 g. Im Laufe dieser Experimente wurden mehrere Versuche gemacht, die vorher erwähnten verschiedenen Ansichten über die Art und Weise der synthetischen Entstehung der Fettsäuren zu prüfen und vor allem festzustellen, ob Kohlehydrat überhaupt das Material ist, aus dem die Fettsäuren gebildet werden. Zu diesem Zwecke wurde in einigen Fällen Glykogen oder Glukose zugesetzt oder auch andere Substanzen, von denen es vermutet worden, dass sie Zwischenprodukte bei der Reaktion sind, während deren Kohlehydrate sich in Fettsäuren verwandeln. Keiner von diesen Zusätzen erzielte jedoch ein regelmässig und beständig vermehrtes Endresultat. Dieses scheint darauf hinzudeuten, dass obgleich Fettsäuren unter obigen Verhältnissen innerhalb der Leber gebildet werden, es doch nicht möglich ist, mit Bestimmtheit darauf hinzuweisen, genau in welcher Weise sie entstehen, noch welches Material als Grundlage zu deren Synthese dient.

Es soll hier daran erinnert werden, was von Magnus-Levy unter ähnlichen Bedingungen beschrieben worden ist, betreffend der Bildung von Essig- und Buttersäure sowohl als Milchsäure innerhalb der Leber. Als Hauptquelle der gebildeten Säuren deutet er auf die während des Versuches verschwindenden Kohlehydrate hin. Gleichzeitig mit der Bildung dieser Säuren wurde Wasserstoff freigemacht. Magnus-Levy vergleicht diese Umwandlungen mit der Buttersäuregärung des Zuckers, der oben erwähnten Nenckischen Hypothese beistimmend, welche letztere er als eine synthetische Reaktion betrachtet, die derjenigen typisch analog ist, bei welcher auch die höheren gradzahligen Glieder der Fettsäurereihe aus Kohlehydraten gebildet werden.

Hahn (11) beschrieb Versuche, in denen die Fettmenge in sterilem Blut sich vermehrte, wenn das Blut bei Körpertemperatur erhalten wurde, in welchem Falle die Hinzufügung von Zucker eine weitere Erhöhung zur Folge hatte.

II. Die Synthese der Fettsäureglyzeride.

Es ist uns bekannt, dass in den Wänden des Darmes eine Kondensation von Glyzerin und Fettsäuren stattfindet, seit Munk bewies, dass Fettsäuren, die in den Darmkanal eingeführt worden, sich in dem Chylus als Glyzeride vorfinden. Dieselbe Reaktion findet wohl auch statt, wenn Fett aus Kohlehydraten synthetisch hergestellt wird. In beiden Fällen scheint das nötige Glyzerin aus einem Material, welches den Zellen in beliebiger Menge bereit steht, gebildet zu werden. Dass dieses Material Kohlehydrat ist, ist vorderhand wahrscheinlich; und die Hypothese, dass die Bildung von Glyzerinaldehyd die erste der Veränderungen darstellt, wodurch Zucker in Fett umgewandelt wird, passt natürlich leicht hinein, um auch die Bildung des Glyzerins aus Zucker zu erklären.

Kastle und Löwenharts Arbeit über die synthetische Wirkung der aus der Leber, den Gedärmen und dem Magen erhaltenen Lipase, welche die Bildung von Äthylbutyrat aus Alkohol und Buttersäure verursacht, lässt vermuten, dass dasselbe Enzym, welches Fett verseift, auch die Kondensation von Glyzerin mit Fettsäuren verursacht.

Wir hätten es hier somit mit einem Enzym zu tun, das dank seiner Reversibilität nach beiden Richtungen hin im Körper physiologisch wirksam ist, welche Möglichkeit von Croft Hill angedeutet wurde als er die reversible Tätigkeit der Maltase feststellte.

Falls dies auch wirklich der Fall sein sollte, so gehört die weitere Frage: auf welche Weise die scheinbare Verrückung des Gleichgewichtspunktes der Reaktion stattfindet, in das Gebiet der Vermutungen. Die ausgedehnte Verbreitung der Lipase im Körper ermöglicht die Voraussetzung, dass der uns bekannte Vorgang, durch den das Fett aus dem Darm resorbiert wird, sich überall wiederholt, wo Fett im Körper mobilisiert wird. Man könnte somit die neutralen Glyzeride als die unbewegliche, die verseiften Fettsäuren als die in Bewegung gesetzte Form desselben Materials ansehen.

III. Die Synthese anderer Verbindungen als der einfachen Glyzeride aus Fettsäuren.

Das in dem subkutanen, serösen und interstitiellen Bindegewebe sich vorfindende Fett besteht aus einfachen Glyzeriden der Stearin-, Palmitin- und Ölsäure und dient nicht zum Gebrauch der Zellen selbst, in denen es aufgespeichert ist, sondern es steht den Teilen des Körpers zur Verfügung, wo beträchtliche Energieumsetzungen stattzufinden pflegen. Ungefähr 12% des getrockneten Myokardiums in Hunden ist, nach Rubow (12) in Äther löslich, und von der getrockneten Nierensubstanz sogar mehr, ungefähr 13—20%.

Diese fettige Substanz enthält jedoch solche Mengen Phosphors, dass bei Annahme, dass derselbe in der Form von Lezithin (mit 3,8% Phosphor) anwesend ist, sich die in dem Herzen vorgefundene Lezithinmenge als ²/₃ des ganzen Ätherextraktes oder 8% des getrockneten Organs berechnen lässt. In den willkürlichen Muskeln des Hundes fand Rubow ungefähr 4% Lezithin, nach dem Gesamtgewicht der Trockensubstanz berechnet.

Heffter (13) fand, dass mindestens die Hälfte des in der Leber vorgefundenen Fettes in der Form von Lezithin anwesend war, und da die von ihm benutzte Methode es ihm wohl kaum ermöglichte, die ganze in der Leber befindliche Lezithinmenge zu extrahieren, so ist obige Angabe noch zu niedrig gegriffen.

Es ist uns jetzt bekannt, dass unter den Fettsäureverbindungen Lezithin keineswegs die einzige ist, die Phosphor enthält und in Äther löslich ist. Somit ist es nicht möglich, die anwesende Menge solcher Verbindungen aus der Menge des in dem Ätherextrakt vorhandenen Phosphors zu berechnen. Es ist jedoch augenscheinlich, dass die Zellen der wichtigsten Körperorgane noch andere Fettsäureverbindungen als einfache Glyzeride enthalten und wahrscheinlich in beträchtlicheren Mengen als diese letzteren.

Das in den Fettgeweben enthaltene Fett ist noch in anderer Hinsicht von dem in den Organen enthaltenen Fett unterscheidbar, nämlich in der Beschaffenheit der Fettsäuren, die an deren Bildung teilnehmen. Es ist bewiesen worden, dass wenn Fettsäuren aus der Leber, dem Herzen, den Nieren, den Leukozyten oder dem Gehirn mit genügender Sorgfalt isoliert werden, dieselben die Fähigkeit zeigen, viel grössere Quantitäten von Jod zu absorbieren, als die aus den Fettgeweben erhaltenen Fettsäuren. Die Jodzahl der aus Bindegewebe stammenden Fettsäuren variiert etwas bei verschiedenen Tieren von ungefähr 40—65. Die Absorptionsfähigkeit der aus Organen stammenden Fettsäuren, die Leber mitinbegriffen (ausser unter gewissen abnormen Verhältnissen) ist immer höher als 100 und variiert zwischen 115—135 (14). Da ein Teil dieser Fettsäuren sich zweifellos in gesättigtem Zustande vorfindet, in welchem sie kein Jod zu absorbieren imstande sind, und da die Jodzahl von Ölsäure 90 ist, so ist es klar, dass grosse Fettsäuremengen anwesend sein müssen, die mehr Jod aufnehmen, als Ölsäure aufzunehmen imstande ist, wie z. B. die zu den Leinöl- und Linolensäurereihen gehörigen Säuren, welch letztere in dem Fett der Fettgewebe nicht vorhanden sind. Die Fettsäureverbindungen, die also im Körper ausserhalb der Fettgewebe anwesend sind, zeichnen sich einerseits durch Phosphorgehalt aus, andererseits durch die Anwesenheit von mehr als einer Doppelbindung in der Kohlenstoffatomkette. Diese beiden Eigenschaften gehen zusammen, da die Jodzahl der aus Lezithin und anderen phosphorhaltigen Fettverbindungen erhaltenen Fettsäuren sich als sehr beträchtlich erweist. So haben z. B. Henriques und Hansen (15) in aus dem Eidotter und Gehirn erhaltenen Lezithin eine Jodzahl von 96—102 gefunden, oder aber, nach Entfernung der festen, gesättigten Säuren, — 154, woraus sie berechneten, dass 25 % dieser Säuren zu der Leinölsäurereihe gehören. Hartley (14) erhielt Lezithin aus Schweineleber durch Ausziehung mit kaltem Alkohol und Fällung durch Aceton aus einer Ätherlösung des Extraktes; die aus dem Acetonniederschlage erhaltenen Fettsäuren zeigten eine Jodzahl von 144, während dieselbe in den gemischten direkt aus der Leber erhaltenen Fettsäuren 129 betrug. Eine Anzahl von Präparaten wurde in zwei Fraktionen geteilt, welche beide in Aceton unlöslich, von denen die eine jedoch in absolutem Alkohol löslich war. Die letzteren Versuche wurden von Kennaway und mir ausgeführt, das Material bestand aus Schweine-, Ziegen-, Hunde- und menschlicher Leber, es ergaben sich die gleichen Resultate: nach Verseifung der Präparate erwies sich die Jodzahl der Säuren als 120—148. Die Fettsäuren, die von Erlandsen (16) aus dem Lezithin und aus dem Diphosphatid Cuorin aus dem Herzen isoliert wurden, ergaben eine resp. Jodzahl von 110—130.

Ein grosser Teil somit der in den Organen enthaltenen Fettsäuren, in der Form von Lezithin oder anderen Phosphatiden anwesend, befindet sich in stark ungesättigtem Zustande. Trotzdem aber scheinen die uns zur Verfügung stehenden Fakta darauf hinzuweisen, dass hierin nicht die ganze Erklärung der hohen Jodzahl der gemischten, aus dem ganzen Organ nach Liebermann dargestellten Fettsäuren liegt, und dass die anwesenden einfachen Glyzeride zum Teil wenigstens von den in den Fettgeweben vorhandenen insofern verschieden sind, dass sie Verbindungen von ungesättigteren Säuren sind. Denn in erster Reihe ist die Jodzahl der aus Lezithin und Cuorin erhaltenen Fettsäuren kaum, wenn überhaupt, grösser als die der gemischten, aus dem ganzen Organ erhaltenen Fettsäuren, von denen ein Teil aus den einfachen, im Organ anwesenden Glyzeriden herstammen muss. Man muss jedoch in Erinnerung behalten, dass die Jodzahl von Fettsäuren, die durch Verseifung von Präparaten des Lezithins oder des Cuorin bei mehr oder weniger vollständiger Isolierung dieser Körper aus dem Gewebe, in dem sie vorkommen, hergestellt worden, uns wohl kaum eine vollständig korrekte Idee von dem Sättigungsgrad dieser selben Säuren innerhalb des frischen und lebenden Gewebes liefern. Die Herstellung des Lezithins und Cuorins macht es notwendig, das Material einer langen Einwirkung von Lösungsmitteln auszusetzen, von welchen es nicht bewiesen ist, dass sie die Aufnahme von Sauerstoff, zu welcher die ungesättigten Säuren so leicht hinneigen, verhindert. Fernerhin ist das Material in mehr oder weniger feuchtem Zustande der Luft ausgesetzt, was ebenso wie die Erhitzung mit Alkali und Alkohol während Verseifung die Jodzahl herabsetzt. Es ist uns wohlbekannt, dass die ungesättigteren Säuren an der Luft vermittelst Oxydation gesättigt werden. Auf diesem Umstande beruht die im Handel übliche Unterscheidung zwischen trocknenden und nicht trocknenden Pflanzenölen. Es ist

Tatsache, dass auch die in den Tiergeweben anwesenden ungesättigten Säuren, wenigstens insofern als Jodabsorption in Betracht kommt, sich in genau derselben Weise verhalten. Erlandsens Erfahrung mit Cuorin illustriert diesen Punkt. Ein Präparat dieser Substanz sofort nach Zubereitung absorbierte 100% Jod; dasselbe längere Zeit über Schwefelsäure als trockenes Pulver aufbewahrt, absorbierte nur noch 22%, während dessen Sauerstoffgehalt sich als 30 statt 21% erwies und die relativen Mengen der anderen anwesenden Elemente unverändert blieben. Diese Veränderungen beschleunigen sich natürlich bei höherer Temperatur. Folgender Versuch beweist, dass der Verseifungsvorgang nicht ohne Einfluss bleibt. Ein Lipoidpräparat aus Leber, durch wiederholte Behandlung mit Aceton von einfachen Glyzeriden befreit, zeigte die Jodzahl 83. Ein Teil des Präparates, welches verseift worden war, enthielt 60% von Fettsäuren, die in Wasser unlöslich, in Petroleum aber löslich waren. Da nun die Jodabsorption der Lipoide gänzlich den in denselben enthaltenen Fettsäuren zuzuschreiben ist, so kann die Jodzahl dieser Säuren im unverseiften Lipoid als 137 berechnet werden. Die direkte Bestimmung derselben in den Fettsäuren nach deren Trennung erwies sich als 120,7. Es steht somit fest, dass die für die in Lezithin und Cuorin enthaltenen Fettsäuren angegebene Jodzahl durchwegs als zu niedrig angenommen werden darf. Die hohe Jodzahl der gemischten direkt durch Verseifung aus einem Organ, der Leber oder dem Herzen dargestellten Fettsäuren ist wohl mehr dem Lezithin und anderen in dem Organe anwesenden Phosphatiden zuzuschreiben als man zu erwarten geneigt wäre, hätte man sich nach den Werten gerichtet, die man nach Isolierung und nachheriger Verseifung dieser Lipoide für deren Säuren erhalten würde.

Wird dagegen die Leber mit kaltem Alkohol extrahiert und der Alkoholextrakt in Äther aufgenommen und wiederholt mit Aceton gefällt, so können sowohl das Lezithin als die Lipoide grösstenteils aus dem Acetonniederschlag entfernt werden. Die übrigbleibenden sowohl in Äther als in Aceton löslichen Substanzen, wesentlich einfache Glyzeride und Cholesterin ergeben nach Verseifung Fettsäuren, welche mehr Jod aufnehmen als diejenigen, welche in dem Fettgewebe enthalten sind. Die folgenden Zahlen wurden von Kennaway und mir aus solchen Präparaten erhalten: (Siehe Tab. S. 370.)

Der Gehalt an Fettsäuren entspricht 95% in Glyzeriden, 70% in Lezithin. Der Phosphorgehalt in Lezithin ist 3,8%.

Es ist somit nicht unumstösslich bewiesen, es scheint jedoch, soweit wenigstens die Leber in Betracht kommt, wahrscheinlich, dass die hohe Jodzahl der in den Organen enthaltenen Fettsäuren nicht gänzlich dem Lezithin und anderen phosphorhaltigen Verbindungen der ungesättigten Säuren, sondern teilweise den in einfacherer Form als Glyzeriden oder Seifen vorhandenen, zuzuschreiben ist.

Alkoholauszug aus Leber (in Äther und Aceton löslich).

Aus	Gehalt an höheren Fettsäuren und Cholesterin (zusammen) nach Verseifung	Phosphorgehalt	Jodzahl der Fettsäuren und Cholesterin zusammen
Schwein I	79 %	0,47	109
Schwein II	95 %	0,23	77
Hund	85 %	0,53	97
Ziege	93 %	0,14	89
Mensch	89 %	—	101

Es frägt sich also zunächst, wie man die Entstehung dieser ungesättigten Fettsäuren in den Organen zu verstehen hat? Entweder kann man sich vorstellen, dass die aus dem Fettgewebe sowie aus der Nahrung stammenden Fette zu den Organen und besonders der Leber hintransportiert werden, um dort einer Veränderung zu unterliegen, vermittelst welcher neue Doppelbindungen in den langen Ketten der Fettsäuren entstehen. Oder es wäre möglich, dass die mehr ungesättigten in den Fettdepots nicht nachzuweisenden Säuren gar nicht aus den gesättigten gebildet, sondern synthetisch aus Kohlehydrat aufgebaut werden. Ob die eine oder die andere dieser Ansichten der Tatsache entspricht, lässt sich einstweilen nicht entscheiden. — Sollte jedoch die oben beschriebene Hypothese der Entstehung der Fettsäuren aus Kohlehydrat richtig sein, so können wir erwarten, solche Säuren mit mehreren Doppelbindungen entstehen zu sehen, ähnlich derjenigen, welche von Hartley in der Leber nachgewiesen worden ist, und die der Formel $C_nH_{2n-8}O_2$ $(n = 20)$ entspricht.

Wenn aber gewisse Versuche, die jetzt im Gange sind, um obige Frage aufzuklären, den Beweis lieferten, dass diese ungesättigten Säuren aus gesättigten, der Nahrung oder den Fettdepots entstammenden gebildet werden, dann mag dieses heissen, dass diese Veränderung der Zusammensetzung der Säuren dazu dient, sie für deren oxydative Zersetzung vorzubereiten, im Laufe deren die langen Ketten Stück für Stück an den Doppelbindungen voneinander gesprengt werden, worauf die Bruchstücke vollständig verbrannt werden.

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The Phagocytosis of so-called neutral substances.

Experiments with Hippomelanin.

By **J. C. G. Ledingham.**

With Plate I

(Eingegangen bei der Redaktion am 2. Juni 1909.)

The ability of the leucocytes and endothelial cells of the vascular walls to take into their interior unorganised or so-called neutral particles like carbon, carmin, etc., when injected into the animal body, has long been recognised, and numerous experiments in vitro have demonstrated similar phenomena.

While the rôle of the blood serum in furthering the phagocytosis of bacteria has been the subject of much study during recent years, observations on similar lines in connection with the phagocytosis of physiologically neutral bodies have been few.

It has however, been shown in the case of carmin (Wright and Douglas), Carbon (Rosenthal and others), Melanin (Dudgeon and Shattock), Milk globules (Neufeld and Händel), that the serum plays a very important part in the phagocytosis of such particles and that in fact they are capable of opsonisation.

Whether the sensitisation of such bodies is strictly analogous to that of bacteria and takes place according to the same laws is a question which cannot yet be decided, especially as the ultimate processes involved in the phagocytic act are not thoroughly understood. The problem which led up to the following experiments suggested itself as a natural corollary to the demonstration of substances in serum which can further the phagocytosis of neutral particles. Can these neutral bodies which are capable of opsonisation in vitro excite the formation of specific antibodies when introduced into the animal body? In other words will the blood serum of the immunised animal acquire a greatly enhanced power of opsonising these particles?

The substance chosen for experiment was Hippomelanin which is readily obtainable from cases of melanosis in the grey horse. The glands at my disposal had been preserved in spirit for many years. They were absolutely black and appeared te contain almost pure melanin with extremely few cellular elements from which the melanin could be freed with great ease. A small portion of the black mass was emulsified with normal saline in an agate mortar. After passage through filter paper the emulsion was centrifugalised and washed several times in order to get rid of any traces of alcohol or adherent organic elements, and finally autoclaved.

The granules were found to be of very uniform size and as Dudgeon and Shattock pointed out, lent themselves readily to accurate quantitative estimation in phagocytosis experiments. As experimental animals, guinea-pigs were chosen, although it would have been interesting in view of the source of the melanin to have employed horses. Three animals in all were treated with injections of melanin suspended in saline (about 4 c. c. at each injection).

In testing the opsonic content of the serum towards melanin during the process of immunisation, human leucocytes were employed and the period of incubation was as a rule 45 minutes.

The results of the various testings of the fresh immune sera will be given as indices, the controls being the sera of normal guinea-pigs of the same weight as the inoculated animals. Experiments made with the inactivated immune sera in order to determine the nature of the antibody developed in the inoculated animals, will then be detailed.

Before the commencement of immunisation the indices of the experimental animals were found to differ only very slightly from one another.

Guinea-pig "A".

	Dates of injection	Dates of testings	Indices
1st	24. April 1907 (subcut^y)	22. April 1907	1,0
2nd	27. „ 1907 „	4. June 1907	3,1
3rd	5. June 1907 „	7. „ 1907	2,9
4th	11. „ 1907 „	14. „ 1907	2,3
5th	19. „ 1907 (intraper^y)	19. „ 1907	2,1
6th	24. „ 1907 „	24. „ 1907	3,1

The animal was killed 5$^1/_2$ hours after the 6th injection. The post-mortem appearances will be detailed later.

Guinea-pig "B".

	Date of injections	Date of testings	Indices
1st	5. June 1907 (subcut^y)	4. June 1907	1,0
2nd	11. „ 1907 „	7. „ 1907	1,0
3rd	19. „ 1907 (intraper^y)	19. „ 1907	0,71
		24. „ 1907	2,1
4th	26. „ 1907 „	26. „ 1907	3,3
		1. July 1907	3,2

Guinea-pig "B" was killed on 1. July. The protocol will follow later.

Guinea-pig "C".

This animal was kept under observation for a period of ten months. The same control animal was employed throughout the whole period.

	Date of injections	Date of testings	Indices
1st	24. June 1907 (intraper^y)	24. June 1907	1,0
2nd	26. „ 1907 „	26. „ 1907	1,7
3rd	1. July 1907 „	2. July 1907	0,86
4th	6. „ 1907 „	16. „ 1907	3,6
5th	16. „ 1907 „	19. „ 1907	1,9
		26. „ 1907	4,4
		29. „ 1907	6,0
6th	16. „ 1907 (last injn.)	26. Nov. 1907	4,2
		29. „ 1907	4,9
		17. Feby. 1908	3,8
		9. March 1908	4,5

It will be apparent from the above tables that in response to the inoculations the sera of all three animals acquired a greatly enhanced power of sensitising melanin. The actual numerical results obtained in two typical testing experiments may here be quoted:

Testing of sera "A" and "B" on 19. June 1907.
Incubation 1 hr. at 37° C.

"A"	17,6	granules per leucocyte
"B"	5,9	„ „ „
Control "a"	8,2	„ „ „
„ "b"	7,9	„ „ „

Testing of serum "C" on 16. July 1907.
Incubation 1 hr. at 37° C.

"C"	16.6	granules per leucocyte
Control	4,6	,, ,, ,,
Normal Saline	1,8	,, ,, ,,

The exact stage at which the index of the inoculated animals reached a level of 2 or 3 cannot be determined with accuracy, but it appears to be about the third or fourth week after the commencement of the injections. When the index has attained this level, further injections have not the effect of raising it much higher except in the case of Guinea-pig "C" where an index of 6 was once reached (on July 29, 1907). This animal received its final inoculation in August 1907 but the serum-testings performed during the succeeding seven months showed that no diminution of the index had taken place. In the absence of stimulation, the maintenance of a high level of immunity during such a long period is remarkable and would appear to indicate that the production of antibodies proceeds at a very slow rate. From our knowledge of the chemical properties of hippomelanin, however, notably its extreme insolubility one might expect that if it had the power of exciting the tissues to antibody-production at all, it would do so only slowly.

At this point it will be convenient to describe briefly the postmortem appearances in the two animals "A" and "B" which were killed at the close of the inoculation-period.

Guinea-pig "A" was killed $5^1/_2$ hours after its final injection.

A brownish-black sausage-shaped mass, representing the curled up great omentum lay along the greater curvature of the stomach. Streaks of melanin granules rested on the surface of this mass and also on the mesentery. The spleen was not enlarged. Films prepared from scrapings of omentum showed large numbers of polynuclear and endothelial cells containing melanin granules in their interior. The phagocytic properties of the latter type of cell were specially conspicuous.

Films of peritoneal fluid showed melanin granules mainly in the polynuclear cells. There was very little free melanin. In films of spleen pulp a few polynuclear and mononuclear cells contained melanin. No melanin was present in the bone-marrow either free or included in cells.

Microscopic sections were made passing transversely through the gastric mucosa and the omental mass. Enormous numbers of phagocytes stuffed with melanin granules were seen but they were not distributed uniformly throughout the thickened serosa. They occurred in clusters in the more thickened portions of the reticulum where fibroblastic proliferation was most marked (vide Fig. I). In these areas the phagocytic cells were almost invariably of the mononuclear type but in many cases it was impossible to determine the shape of the nucleus owing to complete stuffing of the cell body with granules (vide Fig. II). A small quantity of melanin was lying free between the cells.

An interesting feature was the presence of several minute abscesses which were in process of being shut off by fibroblastic capsules. The cells in these abscesses (which were free from micro-organisms) were without exception polynuclear cells in different stages of disintegration. Some still contained melanin granules in their interior. Near the enclosing capsule were larger numbers of mononuclear cells stuffed with granules so that the appearance suggested a transference of the melanin from the disintegrated polynuclear cells to the mononuclear cells at the periphery. Melanin granules were not found in the interstitial tissue of the gastric mucosa. With regard to the granules themselves, no alteration in appearance and shape could be detected microscopically.

Guinea-pig "B".

The post-mortem condition was essentially similar to that of "A". The great omentum formed a sausage-shaped mass along the greater curvature of the stomach. Some enlarged brownish mesenteric glands were present in this neighbourhood.

The liver serosa showed thickened black nodular lymphatic swellings running in the minute fissures and along the free liver edge. Similar engorged lymphatics occurred in the capsules of the spleen and kidneys.

The bronchial and mediastinal glands were also brownish-black on section.

Lymph gland pulp was examined in saline solution and beautiful specimens of melanin-containing cells were seen. They were mainly of endothelial type.

It may be inferred then that the chief storehouse of the injected melanin is the great omentum, the lymph-glands and spleen being only secondary depôts. As to the ultimate fate of the melanin, had the animals been allowed to live longer, it is impossible to decide. The third animal "C" died during my absence and unfortunately no autopsy was made.

Experiments with inactivated immune serum.

It has been shown that the fresh unheated sera of the immunised animals contained, relatively to control sera, a much greater amount of a substance which furthered the phagocytosis of melanin. To this substance we shall give provisionally the name of "melanopsonin". It remained to be determined whether this body behaved towards inactivation and complementing like an amboceptor.

The three following experiments were made with the view of estimating the so-called tropic content of the immune sera. In all three experiments the inactivated immune sera were employed in the undiluted condition.

Experiment I. 7. June 1907.

	Incubation	Granules per leucocyte
"A" inactivated	1 hr. 50 min.	2,3
"A" "	5 " 10 "	5,1
"B" "	1 " 50 "	0,5
"B" "	5 " 10 "	1,4

"B" had received its first injection two days previously to this experiment.

"B" serum contains only about a fourth of the amount of active thermostable substance shown by "A".

Experiment II. 17. June 1907.

Incubation time = 1 hr.

	Granules per leucocyte
"A" inactivated serum of 10. June	4,0
"A" " " " 17. "	4,1
"B" " " " 10. "	1,3
Control serum inactivated	0,9

It will be seen that "A" still contains more than three times as much tropic substance as "B" and about 4—5 times as much as the control.

Experiment III. 26. June 1907.

In this experiment the undiluted fresh and inactivated sera of "B" and "C" were compared.

Incubation time = 1 hr.

	Granules per leucocyte
"B" unheated serum of 26. June	16,2
"B" inactivated of serum 26. June	1,0
"C" unheated serum of 26. June	8.4
"C" inactivated of serum 26. June	1,3
Control unheated serum of 26. June	4,9

Inactivation has apparently a much greater effect on the melanopsonin of "B" than on that of "C", but in both cases the active tropic substance is almost negligible in comparison with the opsonic substance of the fresh serum.

In the next series of experiments the effect of reactivating the heated immune sera by addition of fresh complement will be shown. Before the phagocytic test, normal guinea-pigs serum and inactivated undiluted immune serum were digested together in equal proportions for 30—60 minutes at 37° C. Thereafter, equal volumes of the melanin emulsion, leucocytes, and immune-serum-complement mixture were incubated for 1 hour at 37° C. Appropriate controls were also made.

Experiment I. 29. July 1907.

"C" serum inactivated	— Compl.	(1 in 4)	3,0	granules	per	cell
"C" „ „	— Compl.	(1 in 2)	3,9	„	„	„
"C" „ „	— Compl.	(undiluted)	5,4	„	„	„
"C" serum fresh	— Saline		4,8	„	„	„
"C" serum inactivated	— Saline		0,8	„	„	„
Complement fresh	— Saline		0,8	„	„	„

The effect of complementing the inactivated immune serum is apparent, the maximum phagocytosis being obtained by the use of undiluted complement.

Another series made on the same date with a different melanin emulsion gave the following figures:

"C" serum inactivated	— Compl.	7,1	granules	per	cell
"C" serum fresh	— Saline	7,0	„	„	„
"C" serum inactivated	— Saline	1,6	„	„	„
Complement	— Saline	3,1	„	„	„

The reactivation of the heated immune serum was thus complete.

Experiment II. 26. November 1907.

The immune serum employed on this date was that of "C" at a period of three months after its final inoculation.

Reactivation of the heated immune serum gave an effect markedly exceeding that of the fresh serum alone.

"C" serum inactivated	— Compl.	15,2	granules	per	cell
"C" serum inactivated	— Saline	1,2	„	„	„
"C" serum fresh	— Saline	13,1	„	„	„
Complement fresh	— Saline	3,1	„	„	„
Complement heated	— Saline	0,3	„	„	„

Experiment III. 9. March 1908.

The serum employed was that of "C" drawn eight months after its final inoculation.

Heated complement was employed instead of saline as a diluent so that in all the tubes the concentration of serum (as such) remained the same.

“C” serum inactivated	— Fresh Compl.	8,5	granules per cell
“C” serum inactivated	— Heated Compl.	2,3	„ „ „
“C” serum fresh	— Heated Compl.	7,3	„ „ „
Complement fresh	— Heated Compl.	1,6	„ „ „

The reactivation was again more than complete.

Absorption experiments.

Experiment I.

The following experiment was designed to test whether an inactivated immune serum which had been digested with melanin, could subsequently be complemented.

One volume of inactivated “C” serum was digested with an equal volume of melanin emulsion at 37 ° C. for 1 hr. 35 min. (Fluid “a”).

As control a volume of the same serum was diluted in equal parts with saline (Fluid “b”).

After digestion “a” was centrifugalised and the melanopsonin-content of the resulting supernatant fluid was tested against that of “b”.

Result:

“a”	0,3	granules per cell.
“b”	0,3	„ „ „

Complement was now added to “a” and “b” and digested therewith for 30 minutes at 37 ° C.

The melanopsonin-content of the two fluids was then examined.

Result:

“a”	(Complemented)	1,9	granules per cell.
“b”	„	5,0	„ „ „

Evidently no complementing of “a” had taken place owing to the absorption of the melanin amboceptors by the previous digestion.

Experiment II.

In this experiment heated complement which had been also absorbed with melanin was employed as control.

Inactivated “C” serum and inactivated normal serum (complement) were digested with equal volumes of melanin-emulsion at 37 ° C for 2 hours. The fluids were then centrifugalised and fresh complement added to each in equal proportions.

The melanopsonin-content of the two fluids was now estimated, a fresh melanin-emulsion being used.

Result:

1) Inactivated “C” serum (after digestion with melanin) + Compl. 2,4 gran. per cel.

2) Inactivated Normal (after digestion with melanin) + Compl. 7,8 gran. per cel.

Again no complementing took place in (1) but the figures would suggest the presence of a complement-deviating body in the supernatant fluid after digestion of the immune serum with melanin. Evidently no such body was present in (2).

Another experiment carried out in the same way yielded the values 1,6 and 2,7 for (1) and (2) respectively. These figures are not significant enough but they certainly show that an immune serum which had been digested with melanin, was incapable of being complemented.

Complement-fixation by the haemolytic method.

Two tubes containing respectively inactivated immune serum and heated complement were digested with melanin and fresh complement for 30 minutes at 37° C.

To each tube were now added ox-amboceptor and guinea-pig's corpuscles (2 hours at 37° C).

Definite haemolysis took place in the control tube but none occurred in the tube containing the immune serum, showing that the melanin-amboceptor combination had fixed the complement and accordingly prevented haemolysis.

Summary and Conclusions.

Hippomelanin when injected into the animal body is able to excite the tissues to the production of an antibody having the properties of an opsonin (melanopsonin).

Agglutinins are developed only to a very slight extent, the immune serum attaining an agglutination-titre not exceeding 1 in 20 even after 24 hours contact with the granules.

The immune serum when inactivated loses a large part of the substance which furthers phagocytosis, but the remaining tropic effect is much greater than that of heated normal serum.

When complement is added to the inactivated immune serum, phagocytic values are obtained exceeding those of the fresh immune serum, showing that the immune serum contains a large amount of amboceptor capable of being complemented by fresh normal serum.

No satisfactory explanation can at present be given of the results obtained in the above experiments. That a body so intractable chemically as hippomelanin, should yet be able in the animal body to excite the production of a specific antibody is sufficiently remarkable to warrant a further study of the so-called inert substances.

According to v. Fürth and Jerusalem (1907) in their recent work on hippomelanin, this substance is extraordinarily resistant to chemical agents and by its insolubility in concentrated caustic alkali is readily distinguished from the melanin of epidermal origin and the melanin of malignant tumors. One must always bear in mind, however, the possibility that the body tissues may possess powers of an entirely different order from those of the pathological chemist of dealing with intractable matter like melanin. The following suggestion may at any rate be offered to be at once refuted.

The melanin granules inoculated into the animal body may still have possessed a coating of equine proteid material and the resulting immune body though revealed only by its opsonic property with regard to the melanin granules, may in reality have been an antibody to the foreign horse serum. This suggestion is however unlikely when one considers that the tumors from which the granules were prepared had been kept in spirit for many years and that the infinitesimal quantity of proteid coating the granules could hardly have been potent enough to excite such a marked response as evidenced by the action of the immune serum.

It has been shown by v. Fürth that artificial melanin prepared by acting on tyrosin with tyrosinase corresponds closely in its chemical constitution and properties with hippomelanin. A quantity of this artificial substance is at present at my disposal and it is proposed in future experiments to compare its action in the animal body with that of the naturally occurring hippomelanin.

Zusammenfassung.

Wenn man Hippomelanin dem tierischen Körper (Meerschweinchen) einverleibt, so kann dieses zu Produktion eines Antikörpers im Blutserum anregen, welcher die Eigenschaften eines Opsonins besitzt.

Agglutinine entwickeln sich nur in sehr geringem Grade. Das Immunserum erhält einen Agglutinationstiter, welcher

selbst nach 24 Stunden langer Einwirkung auf die Granula 1 : 20 nicht übersteigt.

Nach Inaktivierung verliert das Immunserum einen großen Teil der opsonischen Substanz, aber die zurückbleibende tropische Wirkung ist noch viel größer als die des inaktivierten Normalserums.

Wenn man dem inaktivierten Immunserum Komplement zufügt, so bekommt man phagocytische Werte, die die des frischen Immunserums übersteigen — ein Beweis, daß das Immunserum Ambozeptoren enthält, die durch frisches Normalserum komplementiert werden können.

Eine zufriedenstellende Erklärung der Resultate der obigen Versuche kann vorläufig nicht gegeben werden.

Daß ein Körper, so unangreifbar wie Hippomelanin, doch fähig sein sollte, einen spezifischen Antikörper zu erzeugen, bietet sicher zureichendes Interesse, um ein weiteres Studium der sogenannten neutralen Substanzen zu rechtfertigen.

Die Versuche werden mit künstlichem Melanin (Wirkung von Tyrosinase auf Tyrosin) fortgesetzt.

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Wright and Douglas (1903), Proc. Roy. Soc., Vol. 72, p. 357.

Dudgeon and Shattock (1908), Proc. Roy. Soc., Series B, Vol. 80, p. 165.

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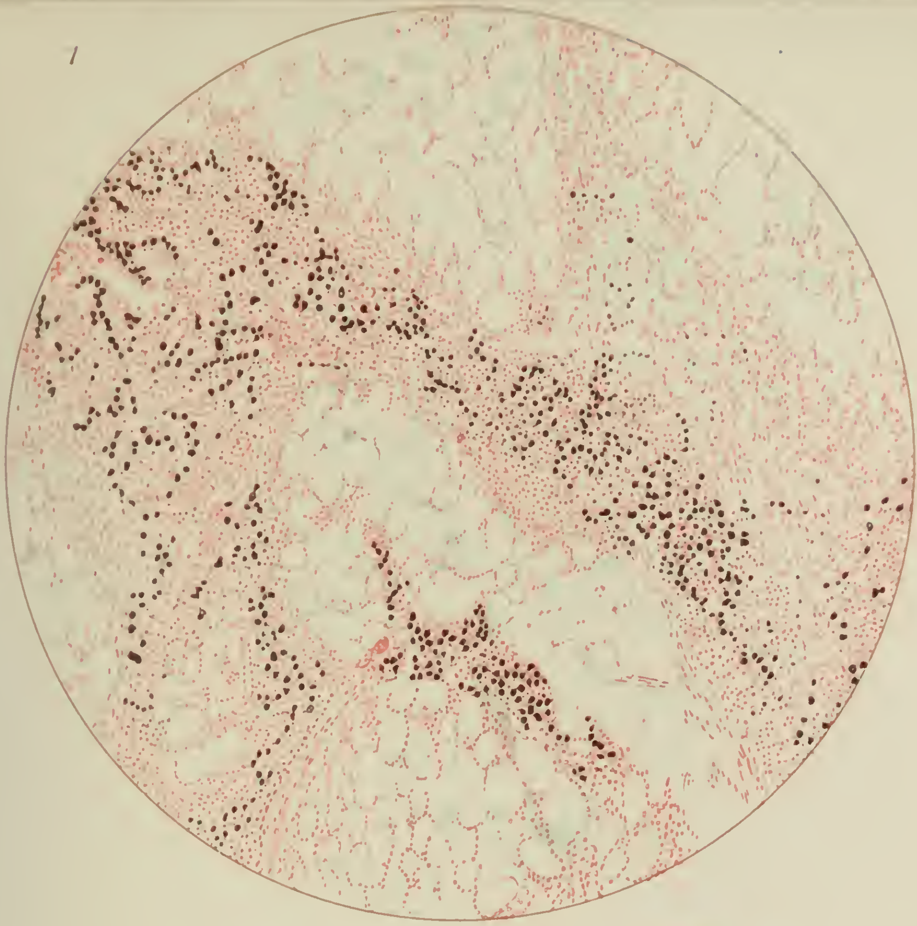
v. Fürth und Jerusalem (1907), Hofmeisters Beiträge, Bd. 10, p. 131.

Plate I.

Fig. I. Section of Great Omentum showing distribution of melanin cells. Leitz, Oc. 4, Obj. 3. Stain Licht-Grün-Neutral-Rot.

Fig. II. Section showing mononuclear cells filled with melanin granules. Leitz, Oc. 4, Obj. $^{1}/_{12}$. Stain Licht-Grün-Neutral-Rot.

1



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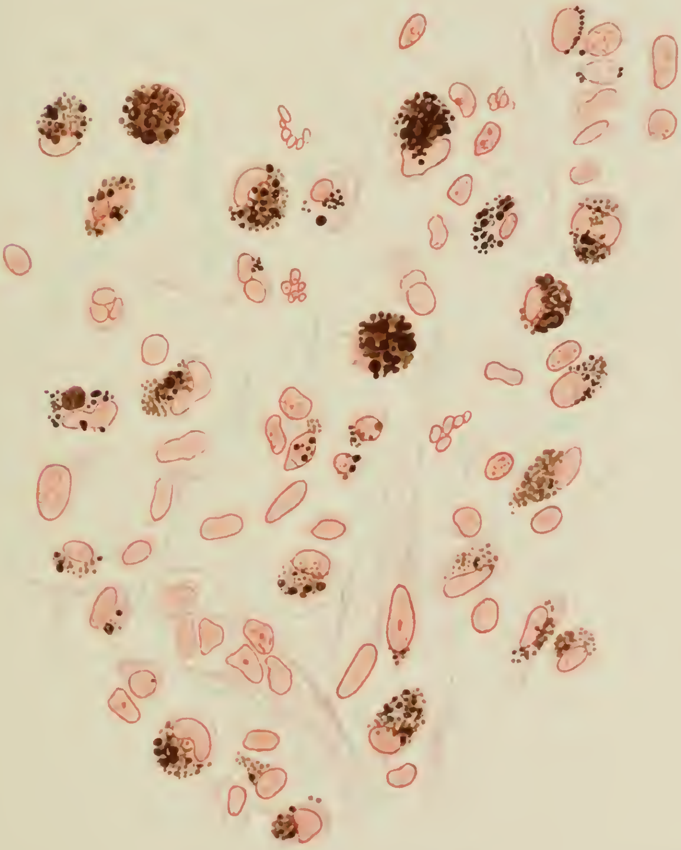

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Observations on the Amœbæ in the Intestines of Persons Suffering from Goitre in Gilgit.

By
Robert McCarrison, M.D., M.R.C.P.Lond.,
Captain Indian Medical Service.

With 24 Text-figures.

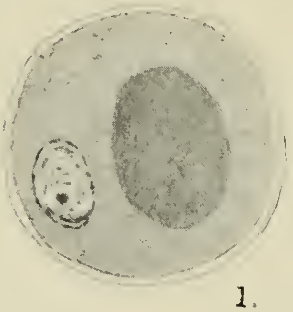
My researches on the ætiology of endemic goitre have led me to the conclusion that in all probability the organism which is responsible for the production of this disease is to be found in the intestinal tract. It is necessary, therefore, to give some account of the Protozoa which are so frequently present in this situation. This paper deals only with the organisms of the Amœba group, and simply gives a brief description and figures of the amœbæ found. It does not claim to be a complete account of their life-histories. While no definite statement can be made as to the pathogenicity of these amœbæ, their possible importance is obvious when it is remembered that goitre is due to an organism carried by water.

The examination of the fresh fæces of goitrous individuals was undertaken solely for the purpose of determining the presence or absence of Protozoa. This object was found to be greatly facilitated by the addition of a small quantity of iodine-water to the specimen, which caused the amœbæ to stand out clearly from surrounding objects. One hundred and three cases were examined in this way; amœbæ were present in eighty-seven. In forty-eight cases they were found in large numbers, in twenty-seven in moderate numbers, and in twelve only after considerable time had been spent in searching for them. The fæces of 101 non-goitrous individuals, living in the same locality, have also been examined. Amœbæ were present in twenty-nine of these; the infection was plentiful in eight, moderate in nine, and scanty in twelve. The typical cysts hereafter described were also found in the only case of goitre from another locality which I have examined.

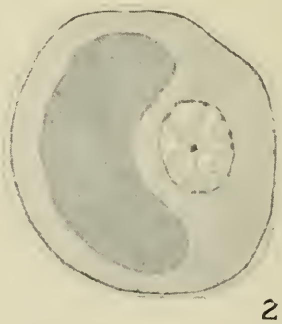
Two distinct amœbæ are found in the fæces; these have not been distinguished in the live state:

(1) A free amœba which proceeds to encyst and develop into a typical 8-nucleated cyst.

Text-fig. 1.[1]



Text-fig. 2.



Text-fig. 1.—Amœba I. Encysted amœba, showing single nucleus and port-wine staining area.

Text-fig. 2.—Amœba I. Encysted amœba. Shows the kidney shape of the port-wine staining area frequently observed.

(2) A free amœba which does not form obvious cysts, but multiplies by division and budding.

In addition, a third amœboid body, enclosed in a characteristic capsule, is also present. Its affinities are not clear, and I can only note its occurrence.

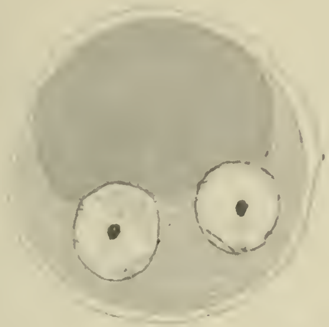
[1] [Figs. 1–7 and 23 are drawn from freehand sketches of the living animals. The preparations were treated with iodine water. Drawings made under Leitz $\frac{1}{12}$ in. oil-immersion, ocular No. 4.

The remaining figures were drawn from fixed and stained preparations, under Zeiss 3 mm. apochromatic homog. oil-immersion, comp. oc. 12 (× 2000). The drawings were made by Miss Rhodes, to whose skill in so accurately depicting the appearances observed I must pay a tribute. The preparations were stained with Haidenhain's hæmatoxylin and Delafield's hæmatoxylin.]

THE AMŒBÆ IN THE FRESH STATE.

(*a*) The free amœbæ.—I can only give a general account of the free amœbæ in the live state, an account which covers both species. In specimens treated with iodine solution the organisms are more or less spherical. Their size varies between 12 μ and 20 μ. Larger forms are occasionally seen. The protoplasm is granular, stains yellow with iodine, and is very rarely vacuolated. A differentiation of the protoplasm into ectoplasm and endoplasm can only be made out in those animals which show pseudopodia, and these are few. The protoplasm contains food-vacuoles and other inclusions. I have never seen any evidence of the ingestion of blood-corpuscles or of epithelium. The nucleus, where observed, is spherical or oval, and is sometimes surrounded by a narrow halo. In the larger organisms it measured 5 μ–8 μ. The characters of the nucleus are very distinct in the two species; they have been studied only in stained specimens.

TEXT-FIG. 3.



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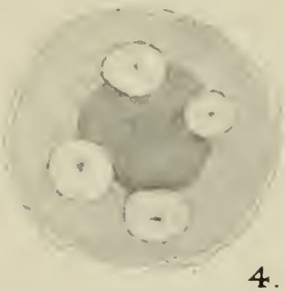
TEXT-FIG. 4.



4.

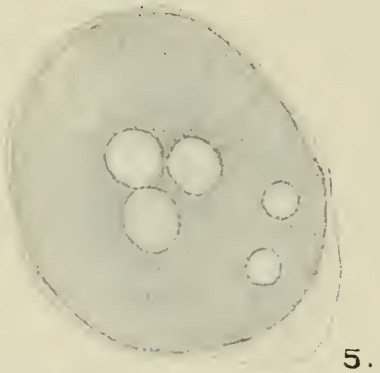
Text-fig. 3.—Amœba I. Encysted amœba. Bi-nucleated stage with large port-wine staining area.

Text-fig. 4.—Amœba I. Encysted amœba. A stage not commonly met with, showing four nuclei and centrally placed port-wine staining area.

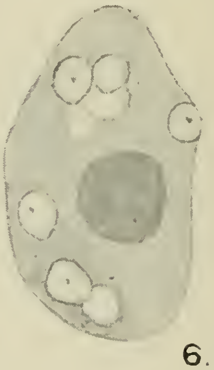
(*b*) The encysted forms.—Live cysts of definite contour are very commonly seen; they represent stages in the life-history of Amœba I, and show different appearances dependent on the length of time the animal has been encysted. These cysts are, as a rule, perfectly spherical, but they may be oval.

Their size varies from 14 μ–20 μ, the latter being the almost constant diameter of the 8-nucleated forms. The cyst-wall varies in thickness, and sometimes it is made out with difficulty. It encloses a yellow staining granular protoplasm, one or more nuclei, and a characteristic port-wine staining area when treated with iodine (text-figs. 1 to 4). The nucleus is spherical, very clearly defined, and shows refractile granules on its surface and in its interior. A well-marked karyosome is usually present. The nucleus varies in size from 2 μ–3 μ in the 8-nucleated cysts to 6 μ–8 μ in the single-nucleated form. The nuclei vary from one to eight in number, dependent on the phase of development of the cyst (text-figs. 1 to 7). The commonest phases met with are those where the cyst contains one, two, or eight nuclei. Cysts showing four nuclei are much less commonly found. The port-wine staining area is present in the majority of all cysts containing one to four nuclei (text-figs. 1 to 4). It is not, as a rule, present in the 8-nucleated cyst; I have only met with it at this stage of development in one instance (text-fig. 6). It is oval, spherical, or kidney-shaped in form, and occupies about one half of the

TEXT-FIG. 5.



TEXT-FIG. 6.



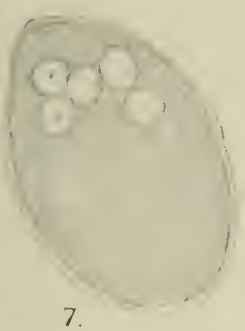
Text-fig. 5.—Amœba I. Encysted amœba. The nuclear division has resulted in the formation of five nuclei; compare text-fig. 17.

Text-fig. 6.—Amœba I. Encysted amœba, 8-nucleated stage, cyst-wall ill-defined; a centrally placed remnant of the port-wine staining area is seen—a very uncommon appearance at this stage.

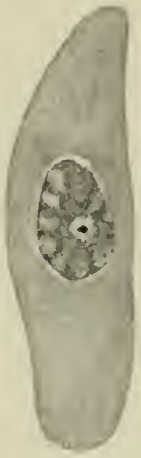
cyst. It usually lies in one hemisphere while the protoplasm is situated in the other, but it may be centrally placed. It appears sometimes to alter its position relative to the nuclei and to the cyst-wall. The port-wine reaction with iodine marks off the area from the rest of the protoplasm in a most distinctive way, for while the protoplasm is granular and stains yellow this area appears to be structureless.

I have never been able to observe a division of the protoplasm around the nuclei in the large 8-nucleated cysts.

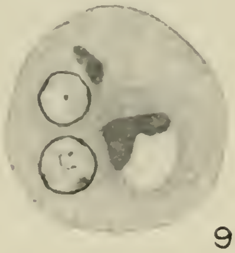
Text-fig. 7.



Text-fig. 8.



Text-fig. 9.



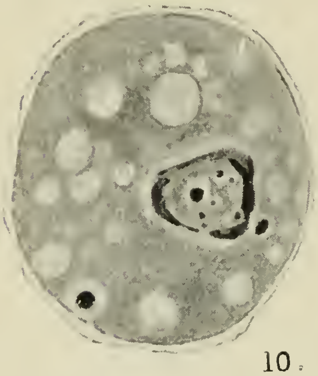
Text-fig. 7.—Amœba I. Encysted amœba. The nuclear division has resulted in the formation of five nuclei; compare text-fig. 17.

Text-fig. 8.—Amœba I. Free amœba. Sideview of organism showing finely granular appearance of protoplasm. The nucleus is seen surrounded by a narrow halo, and shows chromatin more or less evenly distributed with slight massing at four points of the periphery. A central karyosome is seen.

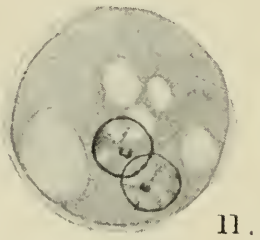
Text-fig. 9.—Amœba I. Unencysted amœba. Probably a stage in simple fission of free amœba. Chromatin massed at periphery of nuclei, which have beaded appearance. Karyosome is seen; nature of included bodies not known.

(*c*) The third organism referred to is less commonly found. The following are its main characteristics in both fresh and stained specimens. In the living state it is seen as a pear-shaped, oval, or spherical body, having a well-defined clear capsule. The protoplasm is granular and stains yellow with iodine. It is split up by a median fissure, on either side of which and approximately at right angles to it is a shorter fissure, which further divides up the protoplasm (text-fig. 23). Movements of the protoplasm and alterations in the position of the nuclei within the capsule are sometimes seen. The nuclei, four in number, usually lie clumped together or in the position shown in the figure (text-fig. 23). They are clear, spherical bodies of uniform size, very sharply defined, and sometimes showing a central dot, features which are well brought out by staining (text-fig. 24). When the organism is stained the capsule is not coloured, and it appears to be open at its broader end. The granular protoplasm sometimes stains so deeply that no structure can be made out. In the more faintly stained animals the protoplasmic fissures referred to can be seen, but not with such distinctness as in the living animal (text-fig. 24). Sometimes the protoplasm, with its contents, is seen contracted up at one side of the capsule. The size of this animal in its longest diameter is fairly constant, and measures in the live state 14μ–15μ.

Text-fig. 10.



Text-fig. 11.



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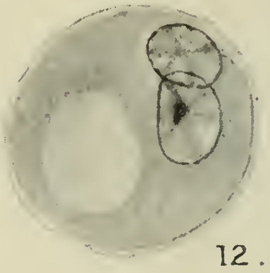


Text-fig. 10.—Amœba I. Encysted amœba. Protoplasm filled with spherical hyaline masses of variable size. Two chromatin masses are seen in the protoplasm. Chromatin heaped up at opposite poles of nucleus; karyosome well marked.

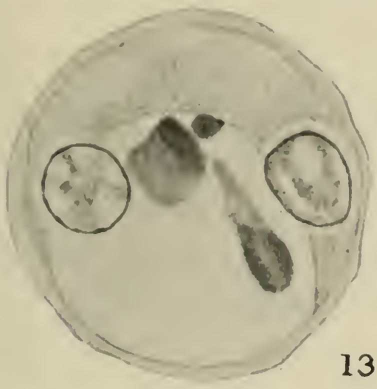
Text-fig. 11.—Amœba I. Encysted amœba. Protoplasm shows hyaline masses of larger size. Nucleus has divided into two. Reticular structure of nucleus and karyosome seen.

Text-fig. 12.—Amœba I. Encysted amœba. Protoplasm shows single hyaline mass. Nuclei as in Text-fig. 11, but of larger size.

The Fixed and Stained Amœbæ.

The material used for this part of the investigation was sent from Gilgit to England in Schaudinn's fixing reagent (saturated watery solution of corrosive sublimate two parts, alcohol one part). The methods of staining employed were Delafield's and Haidenhain's hæmatoxylin. The examination of the material was carried out in Professor Minchin's laboratory at the Lister Institute, to whom I am indebted for help and advice.

Text-fig. 13.



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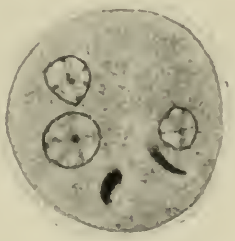
Text-fig. 14.



14.

Text-fig. 13.—Amœba I. Encysted amœba. Shows well-marked cyst-wall. A large hyaline mass occupying about one half of the cyst is seen. Chromatin masses lie above and below this area. Nuclei are seen lying at either side of cyst, each surrounded by halo. Note the reticular structure and granular appearance of nuclei. No karyosome is seen in either nucleus.

Text-fig. 14.—Amœba I. Encysted amœba. One nucleus has proceeded to second division before the other. A common appearance of the nuclei —wheel-like—at this stage is well seen. Two chromatin masses in protoplasm.

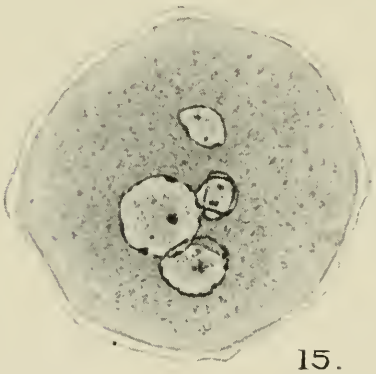
A study of the stained amœbæ shows that there are two distinct species present. In view of the many opinions held with regard to intestinal amœbæ I hesitate to describe these organisms under specific names. Nevertheless, I am inclined to think that Amœba I, which forms the 8-nucleated cysts, is the Entamœba coli, Schaudinn, and that Amœba II corresponds to the Entamœba histolytica, Schaudinn. Certain points in which they appear to differ from the two species of Schaudinn will be referred to in the course of my description.

Amœba I.

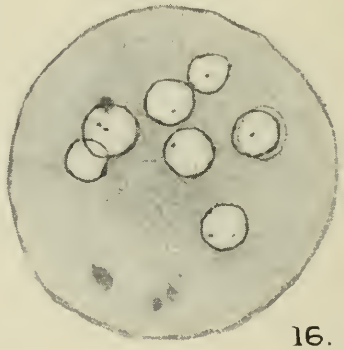
The unencysted amœba usually appears as a spherical body of variable size, ranging up to 20 μ in diameter. The protoplasm is finely and evenly granular (text-fig. 8). I can detect no differentiation into ectoplasm and endoplasm.

In some the protoplasm contains food-material and various inclusions, while in others it appears to be free from extraneous matter. Such inclusions as blood-corpuscles or epithelium have never been met with. The protoplasm rarely shows a vacuole. The nucleus is very distinct, and is usually centrally placed (text-fig. 8). It is commonly surrounded by a narrow but distinct halo. In text-fig. 8 the nucleus appears to lie in a cavity lined by a membrane. Since it has been preserved in a sublimate mixture the appearance may be due to shrinkage of the protoplasm. The nucleus stains deeply; it is rich in chromatin, which in the adult unencysted animal is more or less uniformly distributed throughout this structure. There is often a slight tendency for the chromatin to be

Text-fig. 15.



Text-fig. 16.

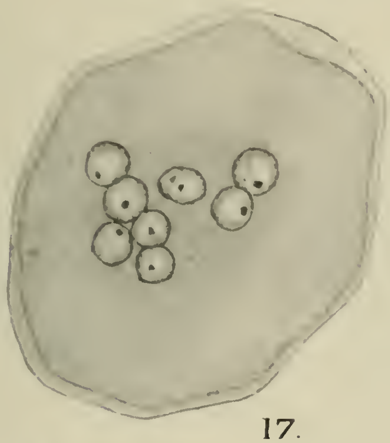


Text-fig. 15.— Amœba I. Encysted amœba. The nuclear division has resulted in the formation of five nuclei.

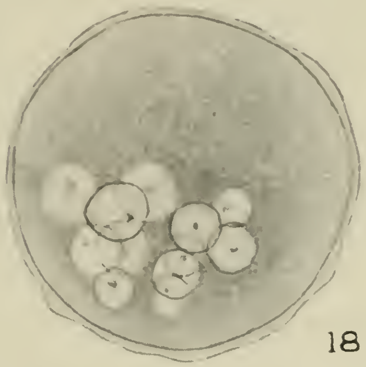
Text-fig. 16.—Amœba I. Encysted amœba. Typical 8-nucleated cyst, of very common occurrence in fæces. Each nucleus is ring-like with a small karyosome in its interior. A few small chromatin masses are seen.

massed irregularly at the periphery of the nucleus. It is generally reticulate in character, and, as a rule, shows a distinct karyosome. I have not been able to satisfy myself as to whether division of the nucleus takes place in the unencysted organism or not. Text-fig. 9 represents an organism of a type only occasionally met with, in which it was impossible to make out the existence of a cyst-wall. This is probably a stage in simple fission of the free amœba. Nevertheless I hesitate to offer an opinion on the point, and simply draw attention to the figure. One point is certain, that in all organisms in which there were more than two nuclei a cyst-wall was always obvious. Unfortunately I have not noted a similar appearance in the living state, but, as I have said, my observations were then only diagnostic.

Text-fig. 17.



Text-fig. 18.

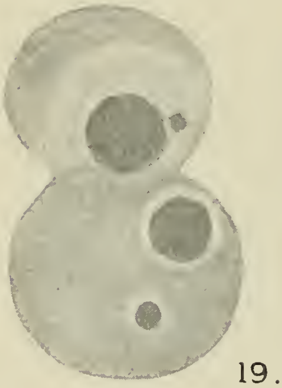


Text-fig. 17.—Amœba I. Encysted amœba; 8-nucleated cyst. The cyst-wall is thicker than in text-fig. 16. The nuclei are very clearly defined and each shows a karyosome.

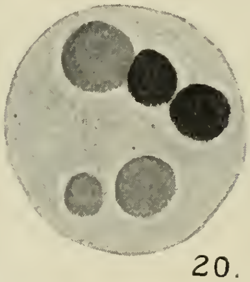
Text-fig. 18.—Amœba I. Encysted amœba. Abnormal form showing twelve nuclei.

The encysted amœba.—The earliest stages of encystment which I have observed are shown in text-fig. 10. The chromatin is heaped up at the periphery of the nucleus, and here and there in the protoplasm dark masses presenting staining reactions similar to those of chromatin are occasionally found. The appearance of the protoplasm is distinctive; it seems to be filled with spherical hyaline masses of variable size. It is difficult to offer an opinion as to the nature of these spherical masses. Such text-figures as 11 and 12 suggest that they gradually fuse, with the ultimate formation of a large, clear hyaline body, as seen in text-fig. 13. This fusion appears to take place in those stages which represent the division of the nucleus after encystation. I am convinced that the clear area (text-fig. 13) is that which gives rise to the characteristic port-wine reaction with iodine water in the living animal. The hyaline body becomes less marked as

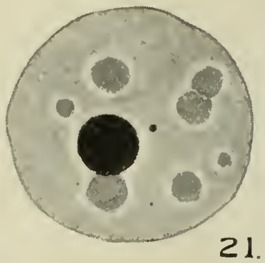
Text-fig. 19.



Text-fig. 20.



Text-fig. 21.



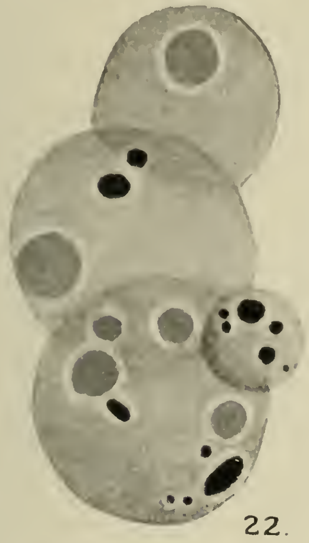
Text-fig. 19.—Amœba II. Two typical organisms. Shows pale staining nuclei surrounded by narrow halo.

Text-fig. 20.—Amœba II. Organism showing three nuclei and two darkly staining bodies. The precise nature of the latter objects is unknown; probably food-material.

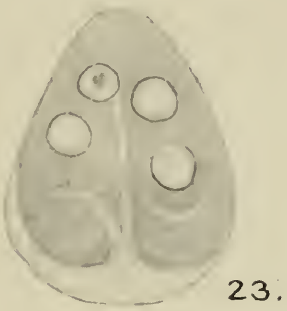
Text-fig. 21.—Amœba II. A multi-nucleated organism. Nuclei of varying sizes. A stage of multiple division.

the further development of the organism proceeds to the typical 8-nucleated form; ultimately it completely disappears. This hyaline body is at its highest development in the late bi-nucleated cyst. I regard this spherical mass as being of the nature of food material, a view which is upheld by Jurgens (**2**). A similar appearance has been described by Wenyon (**3**) in Entamœba muris, and he has considered it to be "of the nature of food products which have not been thrown out of the animal." It is questionable, however, whether the body seen by Wenyon is identical with that which I am describing. Wenyon has found that the refractile body of E. muris "stains feebly, and shows a coarse reticular structure," and that on breaking up "the separate parts shrink to form masses which stain deeply with hæmatoxylin." The hyaline body I have described has not these characters. Schaudinn (4) has described the protoplasm of the encysted E. coli as "divisible into an outer and denser layer containing the nucleus and an inner and more liquid portion,"

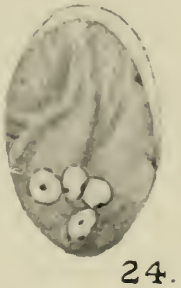
TEXT-FIG. 22.



TEXT-FIG. 23.



TEXT-FIG. 24.



Text-fig. 22.—Amœba II. Group of amœbæ, the result of multiple division; a common appearance.

Text-fig. 23.—The third amœboid body. Shows the characteristic capsule and fissured protoplasm as observed in the fresh state. The drawing also shows a common position of the four spherical nuclei.

Text-fig. 24.—The third amœboid body. Shows the typical appearance of this organism when stained. Note the slightly beaded appearance of the nuclei, the presence of karyosomes, the position of the nuclei, and the manner in which they lie clumped together. The unstained capsule and the fissured protoplasm is well seen.

and Wenyon considers that the more liquid portion probably corresponds to the refractile body of E. muris. It does not, however, correspond to the hyaline body of the amœba under consideration. The characteristic port-wine reaction differentiates it from the yellow-staining granular protoplasm. This hyaline body is a conspicuous feature of the great majority of encysted amœbæ during the earlier phases of their development.

At the time of encystment there is one nucleus present (text-fig. 10). This nucleus divides into two daughter-nuclei (text-figs. 11, 12). I have not been able to trace the complicated series of nuclear changes described by Schaudinn in E. coli and by Wenyon in E. muris as occurring at this stage, though I have seen a large number of amœbæ. The two daughter-nuclei lie most commonly side by side (text-figs. 11, 12). At this stage they vary greatly in size. I have seen them so large their diameter almost equalled half that of the cyst containing them. I have observed in one case a division-figure corresponding closely to fig. 73 of Dobell's paper (**5**); unfortunately the specimen could not be drawn. In text-fig. 13 the two nuclei have separated to either side of the cyst. Division of the two daughter-nuclei takes place, and text-fig. 14 shows that one nucleus has proceeded to the second division before the other. It has been difficult to find examples showing four nuclei; text-fig. 15 shows a further division of the nuclei into five. The ultimate division into eight is shown in text-fig. 16. The cyst-wall eventually becomes thickened as in text-fig. 17. Division of the protoplasm around the nuclei has never been seen. Very rarely a form showing more than eight nuclei is met with (text-fig. 18), but it is so rare that we must regard it as abnormal.

It is evident that the animal here described corresponds very closely to the Entamœba coli of Schaudinn and to the Entamœba muris of Wenyon. The descriptions, however, do not correspond as regards the hyaline body, which is so characteristic of this organism; nor have I been able to trace in it the nuclear changes described by these observers.

Amœba II.

Amœbæ of this species are exceedingly plentiful in some cases; as many as three or four are often found in one field of the microscope. They may occur alone or in association with one or other of those described in the preceding sections.

The animal is usually spherical and of variable size, the forms most commonly met with averaging $15\,\mu$–$20\,\mu$ in diameter. The protoplasm is sometimes divisible into a granular endoplasm with a thin layer of ectoplasm surrounding it. The ectoplasm is most obvious in these rare cases in which protrusions in the form of pseudopodia are seen. Often it is not very clearly marked off. The endoplasm is finely granular and contains extraneous matter; such inclusions as blood-corpuscles or epithelium have never been seen. I have not observed vacuoles. The nucleus in some cases is very difficult to see, presenting as it does staining reactions differing only a little from those of the protoplasm. It is frequently surrounded by a clear halo (text-fig. 19). It is very poor in chromatin and does not appear to possess a karyosome. There is nothing distinctive in the position of the nucleus; it may be central, but is more frequently excentric. It varies very considerably in size. Forms are commonly seen containing two or more nuclei (text-figs. 20, 21). Multiplication is apparently very rapid, and takes place either by a process of simple division or by multiple division of the nuclei with budding of the protoplasm, examples of which processes are shown in text-figs. 19 and 22). This organism does not encyst as does Amœba I. I have been unable to find that it develops cysts such as Schaudinn has described in E. histolytica. It is true that I have frequently found small, dark brown, spherical bodies in preparations where this was the only amœba present; their structure could not be made out, nor could it be shown that they had any connection with this amœba.

This organism resembles closely the E. histolytica of Schaudinn. There was, however, no evidence that patients whose fæces swarmed with this Amœba were suffering from dysentery.

I would also like to point out that the amœba here described is much smaller in size than the Entamœba histolytica of Schaudinn.

Lister Institute,
March 9th, 1909.

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FURTHER OBSERVATIONS ON THE DIFFERENTIATION OF LACTOSE-FERMENTING BACILLI, WITH SPECIAL REFERENCE TO THOSE OF INTESTINAL ORIGIN.

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In a previous paper (1906) I entered a plea for the use of certain tests in the bacteriological examination of waters and food stuffs as it seemed we should thus be enabled to better differentiate the lactose-fermenting bacilli. Such differentiation seems necessary because of the importance attached to *B. coli* as an indicator of faecal contamination, and because of the differences of opinion which exist as to the characters which justify us in identifying a bacillus as *B. coli*.

Thus according to Savage (1906, p. 77) the English Committee appointed to consider the Standardisation of Methods for the Bacterioscopic Examination of Water defined *B. coli* as:

> "A small, motile, non-sporing bacillus, growing at 37° C. as well as at room temperature. The motility is well observed in a young culture in a fluid glucose medium. It is decolorised by Gram's method of staining. It never liquefies gelatine, and the gelatine cultures should be kept at least 10 days in order to exclude a liquefying bacillus. It forms smooth thin surface growths and colonies on gelatine, non-corrugated, growing well to the bottom of the stab (facultative anaerobe). It produces permanent acidity in milk, which is clotted within 7 days at 37° C. It ferments glucose and lactose, with the production of both, acid and gas. The typical bacillus coli must conform to the above description and tests. It generally also forms indole, gives a thick yellowish brown growth on potato (greatly dependent on the character of the potato), sometimes ferments saccharose (about 50 %), changes neutral red (Grübler's) and reduces nitrates, and half the gas produced by it from glucose is absorbable by KOH; and these tests, if time and opportunity permit, may be performed in addition to the foregoing";

and according to Prescott and Winslow (1908, p. 103) the Committee on Standard Methods of Water Analysis of the American Public Health

Association in 1905 drew the following set of diagnostic characters for *B. coli*:

"(1) Typical morphology—non-sporing bacillus, relatively small and often quite thick.

(2) Motility—when a young broth or gelatine culture is examined.

(3) Fermentation of dextrose broth, with the formation of about 50 % of gas, of which about one-third (CO_2) is absorbed by a 2 % solution of sodium hydrate.

(4) Coagulation of milk, with the production of acid, in 48 hours or more at 37° C., either spontaneously or upon boiling.

(5) Non-liquefaction of gelatine.

(6) Production of indole in peptone solution.

(7) Reduction of nitrates."

These two descriptions differ in the importance they attach to the various characters: the American Committee insisting upon the indole and nitrate tests, while the English Committee consider that it is only necessary to perform these tests "if time and opportunity permit." The characters about which they appear to be in accord are: Morphology, motility, the fermentation of dextrose and lactose, the production of acid and clot in milk and the non-liquefaction of gelatine. But even these few have not all been accepted as absolutely necessary; for Prescott and Winslow (1906, p. 104) and Savage (1906, pp. 154 and 157) think that motility is not always present and that its absence is of no significance. There are also differences of opinion with regard to the value of the appearance of the growth on gelatine and on agar.

It seems therefore advisable to go into the question of the weight which each character should be allowed to carry.

TESTS IN COMMON USE FOR DIFFERENTIATION PURPOSES.

Morphology.

B. coli is described as a short bacillus with rounded ends but all sizes may be seen in the same culture.

Buxton (1902, p. 201), speaking of the group of organisms intermediate between *B. coli* and *B. typhosus*, says "Morphologically the intermediates cannot be distinguished among themselves nor with any degree of certainty from *B. coli communis* or *B. typhosus*." Durham (1900—1901, p. 354) says "Speaking generally, morphological characters are not of much value for subdivision of these bacteria" (*B. typhosus*, *B. coli*, etc.). Horrocks (1903, p. 369) describes the morphology of his six groups in the same terms. As his groups include *B. coli*, *B. lactis aerogenes* and others it is obvious he did not find morphology of much differential value. Barber's (1907) studies "On Heredity in certain micro-organisms" also show how much *B. coli* can vary in its morphology. I have several times examined carefully 50—100 film preparations, one after the other, and tried to classify them according to the size and shape of the bacilli, but I have never yet succeeded in separating even one variety from all the others. I am consequently of opinion that no one would reject an organism as *B. coli* simply on account of an "atypical" morphology.

Motility.

It is very difficult to arrive at a conclusion with regard to this character. There is no doubt that an organism may be actively motile in a young (say a 6 hours' broth) culture and yet be quite immobile when examined in a drop of the same culture 12—18 hours later. Sometimes dilution has the effect of starting movements in an apparently non-motile culture. An organism may also be motile when grown at 37° C. or vice versa. One would then be inclined to say that this character varies so much that it can carry no weight one way or the other; and yet no one would accept a motile bacillus as *B. lactis aerogenes* or *B. pneumoniae* (Friedländer). If we allow a value to this character in one case can we consistently deny it a value in a closely allied case. May it not be simply want of knowledge which prevents us forming a just appreciation of it, and would it not be as well to observe this character in each case until we can form a definite opinion about it. A drop of a 6 hours' broth culture placed on an ordinary slide without coverslip and examined, under dark ground illumination, with a $\frac{1}{2}$ inch objective and a × 8 eyepiece will give an excellent idea of the power of movement possessed by an organism. The drop can then be spread, dried and stained in the ordinary way. It seems a pity not to make this observation when it entails so little labour and may eventually prove a necessity.

Broth, bloodserum, agar, potato.

The general opinion is that these media have no value for differentiation purposes.

Litmus milk.

Almost every lactose-fermenter I have tested has produced acid and clot in milk when grown for a sufficient time. All have produced enough acid to cause coagulation on boiling the milk. This medium is used in routine work not for the investigation of enzyme action but merely to observe the production of acid from lactose. Therefore when a pure lactose medium is used it is unnecessary to use milk as well. If milk be used it must according to Biffi (1906) be sterilised always at the same temperature as many bacteria coagulate it when sterilised at 100° C. which will not coagulate it when sterilised at a higher temperature.

Production of acid in litmus whey.

This test has not come into general use, and rightly so in my opinion, as the small amount of information obtained from it is far from being commensurate with the trouble entailed in the production of the medium and the performance of the test.

Production of fluorescence in neutral red media.

So many organisms give this reaction that the tendency is not to lay any stress upon it.

Reduction of nitrites.

This also appears to be a property common to a large number of organisms and no differential value is attached to it. (Savage, 1906, p. 80—Horrocks, 1903, p. 364—Gruber, Th. 1906, p. 656.) I tested some 70 lactose fermenters and non-lactose fermenters and found that all produced nitrites from nitrates as evidenced by Ilosvay's reagent.

Nutrient gelatine.

The appearance of slope or stab cultures depends so much upon the quantity and kind of material used for inoculation that it is difficult to understand how these can be preferred to plate cultures. In the case of the latter the presumption is that each colony starts from a single organism and therefore if there is plenty of room for development any characteristic appearance should be more evident in a colony than in a streak or a stab culture. But the colonies of an organism may vary, even on the same plate, and organisms with different characters may grow alike in colony form (Savage, 1904, p. 358, 1906, p. 81—Horrocks, 1903, p. 369—Radzievsky, 1900, p. 369—von Freudenreich, 1904, p. 408—Leichmann, 1899—Löhnis, 1907, pp. 114 and 115—Gruber, 1906, pp. 655

and 719). Klein (1899—1900, p. 373) says "it is not safe from mere appearances on gelatine to regard particular colonies as those of *B. coli* or its varieties. Such colonies cannot without animal experiment be declared not to be the bacillus of pseudo tuberculosis. Moreover they may be neither *B. coli*, nor its varieties, nor the bacillus of pseudo tuberculosis."

Zlatogoroff (1904, p. 520) thinks that the colonies of *B. pestis* on gelatine resemble those of *B. coli*.

Klein and Houston (1899—1900, p. 601) describe a bacillus which gave typical coli-like colonies on gelatine and appeared in every way typical of *B. coli* except that it did not clot milk and liquefied gelatine after some weeks.

Con and Esten (1904 a) after describing the appearance of the colonies of the *B. lactis aerogenes* group on milk-whey-peptone-gelatine (reaction + 15 to phenolphthalein) say that this group includes several different species. There are at least four different types. Some of the colonies prove to be *B. coli communis*; some to be cocci instead of rods; others to be *B. lactis aerogenes* except that they do not ferment milk sugar; and in a few cases these colonies prove to be different from any of the foregoing. The same writers in another communication (1904 b) state that *B. lactis aerogenes* and *B. coli communis* produce colonies very similar to each other.

Houston (1904, p. 105) says "The picking out of the coli-like colonies for study in pure culture is after all, even to the expert, a speculative venture."

Longley and Baton (1907) in a paper on the determination of *B. coli* in water say "On account of the insignificant value of the test for liquefaction of gelatine in the examination of the Potomac water......it may hereafter be omitted from our routine examinations without introducing any appreciable error."

My own experience is that if a pure culture of an organism be plated it is not at all uncommon to find colonies of more than one kind on the same plate, and that it is useless to attach to "typical" gelatine growths more than a confirmatory value. The real value of a gelatine culture lies in the information it affords us of the power of the organism to liquefy gelatine. Even this value is discounted by the fact that some of these organisms liquefy very slowly—too slowly for this character to help us in routine work.

Production of indole.

Opinions seem very much divided regarding the value of this test. On going through the literature one very frequently meets with the statement that the power of producing indole is a variable character. Most bacteriologists would agree that a typical *B. coli* should produce indole in peptone water and yet few would state definitely that they would reject a bacillus which differed from a typical *B. coli* only in failing to produce indole. It seems to me that this conflict of opinion in part arises from the use of the sulphuric acid and nitrite test, which without doubt does give varying results. On the other hand, if Ehrlich's test (Böhme, 1905, Marshall, 1907, Steensma, 1906) be used it is rare to find a second test yield a result different to the first. When using this test it is best to use cultures which have been grown for 6—7 days (2 or 3 days is not long enough). Then unless there is a distinct red colour produced it is advisable to shake up the culture with amyl alcohol and extract the colouring matter. On more than one occasion I have noticed a faint pink colour appear after the addition of the test reagents, and I have put down the reaction as positive, but on shaking up with amyl alcohol the pink has entirely disappeared—neither the culture nor the alcohol showing the least trace of it. And on the other hand, cultures which did not show any colour after the addition of the test reagents yielded a pale pink to the amyl alcohol.

If these precautions are taken this test seems to be reliable. One cubic centimetre of each solution is sufficient for a test, and if a red colour appears on the addition of the benzaldehyde it is unnecessary to add the potash.

Cultures in which a deep red colour has appeared often show a peculiar result when treated with amyl alcohol. If such a red culture be shaken up with a small quantity of amyl alcohol some of the red colour will be extracted by the spirit. If the coloured spirit be pipetted off, more alcohol added and the shaking up repeated, more of the colour will be removed, and if this process be repeated several times it will be found that the culture has lost its distinct red and become bluish or violet and sometimes almost black.

Fermentation tests.

Glucose and lactose are the only fermentable substances in common use for the differentiation purposes we have in view. *B. coli* is acknowledged by all to decompose both these sugars with the production of acid and gas. Further tests of this kind seem to be considered unnecessary, and Savage (1906, p. 83) may be said to voice the general opinion when after giving a definition of *B. coli* he says: "Organisms with all the above characters whether they ferment saccharose, dulcit, etc. or not can all be spoken of as *B. coli*."

We have now considered all the tests which are usually employed for the purpose of differentiating *B. coli* from other organisms. It remains to see how far these tests will help us in connection with organisms isolated in the present research.

Examination of 497 *lactose fermenting bacilli isolated from various sources.*

In all 76 samples have been studied, the origin of which was as follows:

Human faeces	20 samples	Rain water	1 samples
Human sputum	9 „	Roof washings	2 „
Human pus	1 „	Oats	1 „
Horse faeces	11 „	Crushed oats	1 „
Calf faeces	7 „	Beans	1 „
Goat faeces	2 „	Ear of corn	1 „
Goose faeces	6 „	Bran	1 „
Pig faeces	1 „	Old hay	1 „
Cesspool sewage	1 „	Malt	1 „
Soil	2 „	Baker's yeast	1 „
Pond water	4 „	Cheese (Coulommier)	1 „

The

"Human pus" was the purulent discharge from the operation wound in a case of appendicitis.

"Pond water" was turbid water taken from a pond in the Institute grounds. It receives the washing of a heavily manured flower border and contains hundreds of gold fish.

"Rain water" was rain water caught in a sterile funnel placed in a sterile flask and left out all one night in a field during rainy weather.

"Roof washings" were rain water collected as it flowed from the rain water pipe of a cow shed in an open field.

"Crushed oats"—"Bran." These were samples taken from the bin in which they were stored for feeding horses.

"Oats"—"Beans" were samples of whole oats and beans collected with little risk of faecal contamination, and for which I desire to thank Prof. John Percival, Director, Agricultural College, Reading.

"Old hay" was from the interior of a truss cut from a stack two years old.

"Malt" had been kept in a tin box in the laboratory for quite three years.

"Ear of corn" was picked from a hedge bordering a narrow country lane. It hung about 6 feet from the ground. There had been no rain for about 3 weeks, the dust in the lane was about 1 inch deep and the hedges were covered with it. The ear was dropped into a tube of ordinary nutrient bouillon, incubated over night at 37° C. and plated on bile salt lactose agar.

The method of examination was to plate out either direct from the sample or after preliminary incubation in a liquid medium. Where possible bile salt media (MacConkey, 1908) have been employed because of their inhibiting effect upon many organisms of the air, soil and water, the presence of which would have materially increased the amount of labour necessary for the prosecution of the work.

Cultures were made from single colonies, and these presumably pure cultures were subjected to the tests named. That it is necessary to remember that colonies may be composed of more than one variety of organism was emphasized by the fact that some of these cultures were not pure and had to be replated. The results are given in Tables I and II (pp. 94, 95).

A word of explanation is necessary with regard to the numbers attached to the bacilli in the tables. In previous papers the lactose fermenting bacilli were divided into 4 groups according to the fermentative action on saccharose and dulcit. Thus:

Group I contained bacilli which were saccharose – dulcit –
" II " " " " " – " +
" III " " " " " + " +
" IV " " " " " + " –

If we add, as further tests, the action on adonit and inulin the presence or absence of motility, of indole and of Vosges and Proskauer's reaction, it is possible to form 128 combinations, or to put it another way, we might isolate 128 varieties of lactose fermenting bacilli. But only about $\frac{1}{4}$ of this number have been met with, and so in order to allow for the correct placing of other bacilli 32 numbers have been assigned to each group.

If we study Table I in the light of what has gone before we note that

(1) all these bacilli ferment lactose with the production of acid and gas,

TABLE I. *Characters of the bacilli isolated.*

			Lactose	Litmus milk	Gelatine	Gram's stain	Motility	Indole	Reduction of nitrates	Acidity in litmus whey	Saccharose	Dulcit	Adonit	Inulin	Inosit	Vosges and Proskauer's reaction
Group I, Nos. 1–32	No. 1		+	+	−	−	+	+	+		−	−	+	−	−	−
	,, 2	*B. acidi lactici* (Hüppe)	+	+	−	−	−	+	+	23 $^0/_0$	−	−	+	−	−	−
	,, 3	*B. levans*	+	+	+	−	+	−	+	14 $^0/_0$	−	−	−	+	−	+
	,, 4	*B. Grünthal*, *B. sulcatus gasoformans*, *B. castellus*	⊥	+	−	−	+	+	+	20 $^0/_0$	−	−	−	−	−	−
	,, 5	*B. vesiculosus*	+	+	−	−	−	+	+	25 $^0/_0$	−	−	−	−	−	−
	,, 6		+	+	−	−	−	+			−	−	−	−		+
	,, 7		+	+	−	−	+	−	+		−	−	−	−	−	−
	,, 8	*B. coli mutabilis* (Massini)	+	+	−	−	−	−			−	−	−	−	−	
Group II, Nos. 33–64	,, 33		+	+	−	−	+	+			−	+	+	−	−	−
	,, 34	*B. coli communis*, *B. cavicida*	+	+	−	−	+	+	+	28 $^0/_0$	−	+	−	−	−	−
	,, 35	*B. Schafferi**	+	+	−	−	−	+	+	22 $^0/_0$	−	+	−	−	−	−
	,, 36		+	+	+	−	+	−	+		−	+	−	−	−	−
Group III, Nos. 65–96	,, 65	*B. oxytocus perniciosus*	+	+	+	−	−	+	+	26 $^0/_0$	+	+	+	+	+	+
	,, 66		+	+	−	−	−	+	+		+	+	+	−	−	−
	,, 67		+	+	−	−	−	−	+		+	+	+	−	+	+
	,, 68	*B. rhinoscleroma*, *B. Friedländer*	+	+	−	−	−	−	+	14 $^0/_0$	+	+	+	−	+	−
	,, 69		+	+	+	−	+	−	+		+	+	−	+	−	+
	,, 70		+	+	+	−	+	−			+	+	−	+	−	−
	,, 71		+	+	−	−	+	+	+		+	+	−	−	−	−
	,, 72	*B. neapolitanus*	+	+	−	−	−	+	+	22 $^0/_0$	+	+	−	−	−	−
	,, 73		+	+	+	−	+	−	+		+	+	−	−	−	+
	,, 74		+	+	−	−	+	−			+	+	−	−		−
	,, 75		+	+	−	−	−	−			+	+	−	−	+	+
Group IV, Nos. 97–128	,, 97		+	+	+	−	−	+	+		+	−	+	+	−	+
	,, 98		+	+	−	−	−	−	+		+	−	+	+	+	+
	,, 99		+	+	−	−	−	−	+		+	−	+	+	+	−
	,, 100		+	+	−	−	+	+			+	−	+	−		−
	,, 101		+	+	−	−	−	+	+		+	−	+	−	+	−
	,, 102		+	+	+	−	+	−			+	−	+	−		+
	,, 103	*B. lactis aerogenes*, *B. dysenteriae vitulorum*, *B. capsulatus* (Pfeiffer)	+	+	−	−	−	−	+	30 $^0/_0$	+	−	+	−	+	+
	,, 104	*B. gasoformans non-liquefaciens**	+	+	−	−	−	−	+	12 $^0/_0$	+	−	+	−	⊥	±
	,, 105		+	+	+	−	+	−	+		+	−	−	+	−	+
	,, 106		+	+	−	−	+	+			+	−	−	−	−	−
	,, 107	*B. coscoroba*	+	+	−	−	−	+	+	25 $^0/_0$	+	−	−	−	−	−
	,, 108	*B. cloacae*	+	+	+	−	+	−	+	20 $^0/_0$	+	−	−	−	⊥ −	+
	,, 109		+	+	−	−	+	−			+	−	−	−	⊥	−

Remarks

+ = acid and gas, acid and clot, liquefaction of gelatine etc. as the case may be.

⊥ = acid without gas.

− = no production of either acid or gas.

Inosit. This substance was only used in certain special cases. Nos. 104, 108, 109 gave only slight acid in most cases.

Gelatine. In most cases the liquefaction of gelatine took place very slowly. It is possible that some of those put down as non-liquefiers might have proved themselves liquefiers if they had been kept long enough. All were kept for 2—3 months. Some developed a yellow tint. These were always white at first.

Litmus whey. The cultures were grown for 3 days at 37° C. and were then tested with $\frac{n}{10}$ NaOH. The percentages are given in terms of $\frac{n}{10}$ NaOH, and are meant to be merely average percentages.

* = Both *B. Schafferi* and *B. gasoformans non-liquefaciens* produce usually only a small amount of gas.

			Human faeces	Human pus from appendicitis abscess	Human sputum	Horse faeces	Calf faeces	Goat faeces	Pig faeces	Goose faeces	Cesspool sewage	Soil	Pond water	Rain water	Roof washings	Crushed oats	Oats	Beans	Bran	Cheese	Bakers' yeast	Malt	Ear of corn	
Group I, Nos. 1–32	No. 1		9	—	—	3	2	2	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	21
	,, 2	*B. acidi lactici*	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 3	*B. levans*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0
	,, 4	*B. Grünthal*, *B. sulcatus gasoformans*, *B. castellus*	8	—	—	1	—	—	—	5	4	—	—	—	—	—	—	—	—	—	—	—	—	18
	,, 5	*B. vesiculosus*	33	5	1	—	—	—	3	1	—	1	—	—	—	—	—	—	—	3	—	—	1	48
	,, 6		—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 7		1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	2
	,, 8	*B. coli mutabilis* (Massini)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0
Group II, Nos. 33–64	,, 33		—	—	—	5	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	9
	,, 34	*B. coli communis*, *B. cavicida*	37	—	3	15	3	13	1	3	1	—	—	2†	—	4	—	—	—	—	—	—	—	82
	,, 35	*B. Schafferi*	11	—	—	1	—	—	3	—	—	—	—	—	—	—	—	—	—	1	—	—	—	16
	,, 36		1	—	—	1*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	3*	—	8
Group III, Nos. 65–96	,, 65	*B. oxytocus perniciosus*	1	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	3	—	—	—	8
	,, 66		—	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
	,, 67		1	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
	,, 68	*B. rhinoscleroma*, *B. Friedländer*	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 69		—	—	—	3*	—	—	—	—	—	—	—	—	6*	—	1	—	—	—	—	—	—	10
	,, 70		—	—	—	—	—	—	—	—	—	—	—	1*	—	—	—	—	3	—	—	—	—	4
	,, 71		42	5	—	32	30	—	—	9	—	6	3	1	5	1	—	—	—	—	4	—	5	143
	,, 72	*B. neapolitanus*	15	—	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	17
	,, 73		—	—	—	1*	—	—	—	—	—	—	1*	—	7*	—	1*	3*	—	—	—	6*	5*	24
	,, 74		1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 75		—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	1
Group IV, Nos. 97–128	,, 97		1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 98		—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
	,, 99		—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
	,, 100		1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
	,, 101		1	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	5
	,, 102		—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	2
	,, 103	*B. lactis aerogenes*, *B. dysenteriae vitulorum*, *B. capsulatus*	8	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—	—	—	15
	,, 104	*B. gasoformans non-liquefaciens*	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	2
	,, 105		—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	7	—	—	—	9
	,, 106		2	—	1	1	—	1	—	4	—	—	—	—	—	—	—	—	—	—	—	—	—	9
	,, 107	*B. coscoroba*	1	—	—	—	1	—	—	1	—	3	—	—	—	—	—	—	—	—	—	—	—	6
	,, 108	*B. cloacae*	—	—	—	2	—	—	—	—	—	1	5	3	3	—	—	—	—	—	—	—	—	14
	,, 109		2	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	2	3	—	—	9
			178	10	13	67	40	17	7	23	6	16	11	7	31	5	4	3	3	26	10	9	11	497

* Yellow liquefier. † Yellowish.

(2) they produce acid and clotting in milk,

(3) the majority do not liquefy gelatine, and those which do so liquefy so slowly that they would in routine work be classed as non-liquefiers,

(4) all are Gram-negative,

(5) the motility and indole tests are positive in some cases and negative in others,

(6) in all cases which have been tested nitrates were reduced to nitrites.

The morphology is not noted in the Table, because it proved not to be of any differential value.

Now, according to the tests usually employed, all these bacilli would be classed as *B. coli*. But if we kept them long enough we should find that in several cases gelatine was liquefied and we should be in the position of having classified a liquefying organism as *B. coli*, though all bacteriologists are agreed that *B. coli* is not a liquefying organism.

It has been suggested that the error from this cause is so small that it may be neglected, but an enumeration of the liquefiers in Table II shows that the error would be more than 10 °/₀ and therefore cannot be ignored. It follows that the present methods of differentiation are not adequate and that a change is necessary.

In a former paper I proposed that we should omit the observation of the (1) characters of the growth on gelatine, (2) action on milk, (3) action on glucose, (4) action on neutral red, and (5) the indole test, and that we should substitute for them (1) the action on dulcit, (2) the action on adonit, (3) the action on inulin and (4) Vosges and Proskauer's reaction.

Further experience has only confirmed me in this opinion except as regards the indole test and Vosges and Proskauer's reaction. With regard to the fermentation reactions it is necessary to remember that some bacilli act slowly and that, in consequence, we may be premature if we draw conclusions after the cultures have been incubated for only 24, 48 or even 72 hours. I have changed my opinion of the indole test because an extensive use of Ehrlich's reaction has shown me that this test cannot be omitted with safety.

Vosges and Proskauer's reaction.

I have used this reaction for some years, and it seemed to be reliable and valuable until at the end of 1907 I isolated organisms which gave sometimes a negative or doubtful reaction and sometimes a positive one.

Such results have weakened my faith in this test and yet I cannot say it is of no value as it certainly is not given by every organism. For instance, in 1905 I isolated from an ear of corn 11 lactose fermenting bacilli of which 10 agreed as regards motility and fermentation reactions. These differed in that 5 liquefied gelatine, gave Vosges and Proskauer's reaction and did not produce indole, while the other 5 did not liquefy gelatine, did not give Vosges and Proskauer's reaction but did produce indole. These cultures were kept and tested from time to time and always gave identical results until September 1908 when one of the non-indole-producers gave a positive indole reaction. This culture was at once plated and 6 colonies were subcultured and worked through the various tests. All were motile and all gave the same fermentation reactions as before, but 4 were indole negative and 2 were indole positive. The indole-negative culture gave Vosges and Proskauer's reaction, while the indole-positive ones did not. In this instance Vosges and Proskauer's reaction was of distinct value in confirming the presence of contamination.

No one of these tests is of much value by itself. They must be considered together, each one merely forming one link in the chain of evidence. An example may perhaps make my meaning clearer.

The *B. oxytocus perniciosus*, No. 65, Table I, would according to the usual tests be classed as a non-motile *B. coli*. But it liquefies gelatine, taking perhaps 8—12 months to liquefy ½ inch of the medium. So unless we use further tests we should classify this bacillus incorrectly. When however we study the series of tests given in Table I we observe that it

(1) gives a positive reactive with *all* the fermentable substances used,

(2) produces indole,

(3) is non-motile,

(4) gives Vosges and Proskauer's reaction.

If any reliance is to be placed upon these tests we ought to find that bacilli having these characters liquefy gelatine. This has in my experience proved to be the case.

I isolated from soil several organisms which were *B. oxytocus perniciosus* so far as was evidenced by fermentation reactions, indole and motility. But they appeared to be non-liquefiers. This led me to try what may be termed the "massive inoculation" test for liquefaction (MacConkey, 1906 a). A positive result proved that these bacilli were in truth *B. oxytocus perniciosus* and at the same time afforded evidence in favour of the value of this series of reactions.

One might multiply examples of this kind, but there seems to be no real advantage to be gained thereby. It will suffice to say that as regards *B. cloacae* these results have been confirmed by Ferreira, Horta and Paredes (1908).

Coming now to Table II we are struck by the distribution of the bacilli isolated. If all these organisms were the same bacillus we might reasonably expect that they should be distributed fairly evenly throughout the samples. But such is not the case.

The *B. levans* for instance is so rare that it has not been met with once in 497 bacilli, and yet *B. levans* has been stated to be identical with *B. coli* (Papasotiriu, 1902).

On the other hand 7 of the varieties given in Table I claim 87 °/₀ and 3 varieties 62 °/₀ of the 178 bacilli obtained from human faeces, etc., and of the 154 organisms with an origin in animal faeces 68 °/₀ belong to 2 varieties and 46 °/₀ to a single variety. If we consider the 497 bacilli as one whole we find that 54·9 °/₀ of them belong to one or other of 3 varieties. If all these bacilli are *B. coli* we must allow that certain varieties of this organism are very common in faeces and material exposed to faecal contamination, while certain other varieties are rare in faeces and more common in other materials.

This suggests that the term *B. coli* as at present used is not a happy one, it is too comprehensive, and it would be better to avoid using it. There can be little doubt that all these bacilli have already been isolated and partially described under some name, but I have found it impossible to obtain more than a few named cultures. For the others we must be content to use numbers until we find out the names by which they should properly be known.

I think I have now brought forward sufficient evidence to justify me in once more urging the adoption in routine work of the series of tests recommended in this paper.

Only two further points need be touched upon. Firstly objection may be taken to my classification on the ground that the fermentation reactions are not stable. Some workers, Klotz (1906), Reevis (1908), Twort (1907), seem to consider that these reactions are too inconstant to be of use in classification, but the work of Savage (1906), Horrocks (1903), p. 370, MacConkey (1905, p. 356, 1906 b, p. 399), Villinger (1894), and Benczur (1908), is evidence in favour of the reliability of these tests.

Nor must it be forgotten that this very kind of reaction is considered of great value in the differentiation of *B. coli*, *B. typhosus* and *B. enteritidis* (Gaertner). If they are looked upon as reliable enough for this purpose why should they not be equally of value in separating *B.*

coli from *B. lactis aerogenes* and similar organisms. To say that an organism which does not decompose lactose is not a *B. coli* and then to say that the decomposition or not of some other substance, say dulcit, is of no value in separating *B. coli* from another lactose fermenter is, to say the least, inconsistent.

But there is no need to labour this point. From a practical standpoint it matters not whether these organisms are different bacilli or one bacillus in various guises. If organisms isolated from a certain material (A) give in the majority of cases a certain series of reactions (a_1, a_2, a_3), and if organisms isolated from some other material (B) give in the majority of instances a different series of reactions (b_1, b_2, b_3) then we would be justified in associating the series (a_1, a_2, a_3) with the material (A) and the series (b_1, b_2, b_3) with the material (B); and if we found both the (a_1, a_2, a_3) series and the (b_1, b_2, b_3) series given by organisms isolated from (B) we could presume that some of (A) had become mixed with (B). It might be that both series of reactions were given by the same organism and that the difference was simply the effect of environment. This would not affect the conclusion. It would only show that the organism had not been long enough in (B) to have its (a_1, a_2, a_3) series altered by the changed environment. Secondly it may be asked: "Why limit oneself to these few reactions? Why not use more fermentable substances and have still better differentiation?" I can only answer that I have not found any real advantage in so doing.

We all of us always wish to identify organisms as accurately as possible, in as short a time as possible, and with as little trouble as possible. This desire with regard to time and trouble is intensified when it is a question of routine work. The exigencies of this class of work must always be borne in mind. With this end in view I have given a thorough trial to the following substances:

Glucose—laevulose—galactose—lactose—maltose—mannose—arabinose—raffinose—saccharose—mannit—dulcit—adonit—quercit—erythrit—inosit—sorbit—glycerine—arbutin—salicin—amygdalin—α-methyl-glucoside—inulin—dextrin—and starch;

and I have come to the conclusion that for the purpose of differentiating the lactose fermenters we can be content to use lactose, saccharose, dulcit, adonit, inulin, inosit, and may be mannit[1]. It does not seem worth while using any of the other substances mentioned: for quercit and erythrit remained unaffected when tested with about 100 organisms taken at random; and the others did not afford any more information than is to be gained by using the substances mentioned. These together with the indole test, the observation of motility and perhaps Vosges and Proskauer's reaction will enable us to travel a fair distance on the road to our goal.

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[1] It may become necessary to use mannit because there is a bacillus which ferments glucose and lactose but not mannit.

The method of procedure suggested is that a sloped agar tube should be inoculated from a single colony on a plate, the growth being rubbed all over the surface of the medium and in the water of condensation. After 4—6 hours' growth at 37° C. a drop of the condensation water can be examined to ascertain the presence or absence of motility. After 24 hours' incubation at 37° C. a good loopful of the growth is put into tubes of gelatine, lactose, saccharose, dulcit, adonit and inulin. The agar tube is returned to the incubator, together with the rest of the tubes, and is used later for the indol test. An inosit tube and a glucose tube (for Vosges and Proskauer's reaction) may be inoculated at the same time as the others, or these two may be used as confirmatory tests. Vosges and Proskauer's reaction may be tested for at the end of 4 days. The other tubes should be kept under observation as long as there is no change in the reaction of the medium.

In the course of this work several bacilli have been isolated which have not been included in the results given above. All these bacilli will be found in the subjoined Table III which gives their characters.

CONCLUSIONS.

It has been shown that the tests at present in general use do not allow us to differentiate adequately the lactose fermenting bacilli from each other. It has also been shown that by the substitution and addition of certain other tests we shall gain in accuracy with little increase in labour, and that we shall thus have a fair prospect of being able to pick out those organisms which are most closely associated with faeces and put the bacteriological examination of water supplies upon a firmer basis than that upon which it stands at present.

Note.

While this communication was in the press my attention was directed to a paper by Bergey and Deehan on "The Colon-aerogenes

TABLE III.

Source	Lactose	Saccharose	Dulcit	Adonit	Inulin	Indole	Motility	Milk	Gelatine	Glucose	Mannite	
Human faeces	–	–	–	–	–	+	+	⊥	–	...	...	
,, ,,	–	–	⊥	–	–	–	+	⊥	–	⊥	⊥	
,, ,,	⊥	⊥	⊥	–	–	–	+	...	–	...	...	
,, ,,	–	–	–	–	–	+	+	alk.	–	+	–	Case of typhoid.
,, ,,	–	–	–	–	–	–	+	alk.	–	–	–	
,, ,,	⊥	–	⊥	–	–	+	–	+	–	...	...	
Human sputum	–	–	–	–	–	+	+	sol. of casein	+ green	...	...	Only organism found in the sputum of the same person on two occasions at two weeks' interval.
,, ,,	–	–	–	–	–	–	–	–	–	...	...	
,, ,,	–	–	–	–	–	+	+	+	+ yellow	+	–	
,, ,,	–	–	–	+	–	–	–	+	–	⊥	⊥	
Horse faeces	–	+	+	–	–	+	+	⊥ alk.	–	...	...	1000 per gramme.
,, ,,	⊥	–	⊥	–	–	+	–	+	–	...	...	100,000 per gramme.
,, ,,	–	–	–	–	–	+	–	...	–	+	–	
Cesspool sewage	–	+	+	–	–	+	+	–	–	...	...	
Pond water	–	⊥	–	–	–	+	+	+	+	+	⊥	*
Rain water	–	–	–	–	–	–	–	⊥ sol. of casein	–	–	...	
,, ,,	–	⊥	–	–	–	–	+	⊥	+ yellow	...	...	
Soil	–	–	–	–	–	+	–	alk.	–	+	...	
Old hay	⊥	–	⊥	–	–	–	+	+	+ yellow	...	...	
,,	⊥	–	–	–	–	+	+	+	+ yellow	⊥	...	
Cheese	–	⊥	–	–	+	–	+	+ alk.	–	...	...	
,,	–	–	–	–	–	+	+	alk.	–	...	...	
Faeces & tap water	–	–	–	–	–	–	+	alk.	–	–	...	
,, ,,	–	–	–	–	–	–	+	+	+	–	–	
,, ,,	⊥	⊥	–	–	–	+	+	+	+ yellow	...	...	
,, ,,	⊥	+	–	–	–	+	+	+	+	...	...	*

+ = production of acid and gas, acid and clot, or liquefaction of gelatine as the case may be.
⊥ = production of acid only, no gas. – = neither acid nor gas produced.

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* It has been my experience that in cases of this kind (where an organism produces acid and gas in one medium and apparently only acid in another medium) under proper subcultivation the organism will produce gas also in the second medium. The last organism in the Table could I think have been made to produce gas in lactose. But where an organism produces only acid in several media, *e.g.* the 3rd organism in Table III, then I have never succeeded in inducing it to produce gas in any one of them.

Group of Bacteria" in the *Journal of Medical Research*, Vol. XIX. No. 1 (July, 1908).

These workers examined 50 samples of milk, 1 sample of Kefir and 8 samples of sewage. They made use of the tests advocated above and their results cause them to express opinions which are in complete agreement with mine as to the value of these tests. They attach importance to the gas amount and gas ratio. I have not referred to these two tests because they have been shown to be unreliable by Longley and Baton (1907).

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Observations on the Flagellates Parasitic in the Blood of Freshwater Fishes. By Prof. E. A. MINCHIN, M.A., V.P.Z.S.

(Plates I.–V.*)

Introductory.

The trypanosomes and trypanoplasms of freshwater fishes have been studied by a number of naturalists abroad, and have been the subject of several important memoirs, more especially by Laveran & Mesnil, Léger, and Brumpt in France, and by Keysselitz in Germany. In this country, however, little attention has been paid to them. I was therefore glad to avail myself of the exceptional opportunities offered by the Sutton Broad Laboratory, to study the parasites of the fishes in the Norfolk Broad. I desire to take this opportunity of expressing my warmest thanks to my friends Messrs. Eustace and Robert Gurney for their kindness in putting at my disposal the resources of their picturesque and well-equipped laboratory, and for much help during my stay there. I spent portions of my summer vacations at the laboratory, about three weeks in August and September 1907, and five weeks in the same months in 1908, and during these periods I occupied myself almost entirely with these parasites. Although I have only touched the fringe of the question so far, and there is still everything to be discovered and worked out concerning the all-important question of the transmission of these parasites, I thought it worth while to publish my investigations at the point they have reached, leaving the subject in a condition in which it can be taken up again and carried further by myself or by anyone else working in the same region.

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* For explanation of the Plates see p. 29.

Methods and Technique.

In examining the blood of fishes for the parasites, it is not difficult, when dealing with a fish of fair size, to take the blood from the gills of the fish without materially injuring it. There is always a risk, however, that the blood obtained in this way may contain a certain amount of water mixed with it. To obtain good smears I found it best to sacrifice the fish. Having killed it by a smart blow on the head with a blunt instrument (best a stout rod of hard wood) I opened up the pericardial region and took the blood from the heart by means of a fine capillary glass tube, thrust through the wall of the heart; the blood was allowed to run up into it, and then blown on to the slide or coverslip. The whole process has to be done quickly, since fish-blood coagulates very rapidly. Fish which are used in this way for making blood-smears suffer no detriment in respect to their culinary properties and need not be wasted.

Much has been written lately about methods of fixation of these blood-parasites, especially with regard to the procedure most in vogue, of drying smears of the blood. That the process of drying affects the minute structural details cannot be doubted and can be demonstrated easily. It should, however, be pointed out that the effects of drying, so far as trypanosomes are concerned, differ greatly according as the drying is done before, or after, fixation with some histological reagent. Very instructive in this respect are the species of the genus *Trypanoplasma*. Their soft protoplasmic bodies become greatly deformed if dried before fixation; in this respect they contrast strongly with the species of *Trypanosoma*. In some fishes, for example the pike, tench, and bream, it is common to find both trypanosomes and trypanoplasms in the blood; in smears dried before fixation, the trypanosomes may be found quite satisfactory in form and general structure, while the trypanoplasms side by side with them are deformed almost beyond recognition; compare figs. 25, 26, and 39, showing trypanosomes and trypanoplasms of the pike, from the same slide; figs. 32, 53, 54, showing similar conditions in the blood of the bream. In some cases, however, a trypanoplasm may, apparently, flatten down evenly and thus give a fairly reliable representation of its natural form and structure (compare figs. 37 and 38, from the same slide); such cases, however, are in my opinion to be regarded as accidental and exceptional, and the rule is that trypanoplasms when dried become much deformed. It is therefore a matter of astonishment to me that Keysselitz should have relied so much on material dried before fixation for his lengthy and detailed study of the life-history of *Trypanoplasma cyprini* ("*borreli*"). It is, perhaps, for this reason that Keysselitz was unable to distinguish more than one species of *Trypanoplasma.*

The deformation subsequent on drying is entirely avoided if the smear be placed as quickly as possible, immediately after smearing and before any appreciable amount of drying has taken place, into a stoppered tube or bottle containing a few drops of osmic acid, 4°/₀ solution. It should be exposed to the vapour for 30–60 seconds, and then transferred, with or without drying, to absolute alcohol. After the fixation with osmic vapour the drying does not deform even the trypanoplasms, and the natural form, size, and appearance of these flagellates are, in my opinion, better preserved by this method than by any other. The osmic-fixed smears stain well with Giemsa's stain, but are apt to stain rather too darkly and, in the case of large fleshy forms of the parasites, to become very opaque. Speaking generally, they should be stained for a much shorter time than the preparations dried off before fixation.

The weak point of the preparations stained by the Romanowsky method, whether dried before or after fixation, or not dried at any time, lies in its effects on the nuclear apparatus. I hope to discuss this point in greater detail in a memoir which I am preparing on the structure of *Trypanosoma lewisi.* I will say here only that the Romanowsky stain, in its various modifications, gives results with regard to nuclear structure which are not capable of uniform interpretation, and which, in my opinion, are untrue and misleading. I believe the defects of the stain to be due principally to the fact that the red dye or dyes in the stain are precipitated not only in, but around, certain objects in the preparations; with the result that the kinetonucleus, for example, appears many times its real size, while in the trophonucleus minute, almost ultramicroscopic granules become enlarged to coarse granules obscuring the true structure. This effect is not due to the drying, since it is to be observed in preparations stained by the Romanowsky method and mounted in Canada balsam without being dried at any stage of the process. I believe, however, that the process of drying, whether before or after fixation, may and does add greatly to the falsity of the nuclear results obtained by the stain. While osmic vapour fixes very perfectly the cytoplasmic portions of the body, it apparently leaves the nuclear constituents unfixed and in a fluid condition. Hence, while the general structure of the body is very perfectly preserved, even when dried, after osmic fixation, the nucleus remains in a fluid state and liable to deformation. That, at least, is the impression which a comparison of different methods gives me.

For interpretation of the results obtained by the Romanowsky stain, it is very instructive to compare the totally different results obtained by staining the same objects with Heidenhain's iron-hæmatoxylin. For this it is necessary, however, that the smears should be suitably fixed and that they should have never been dried. I have tried various fixatives and get the best results with sublimate-acetic ($HgCl_2$ saturated in water 95 volumes, glacial acetic 5 volumes), Schaudinn's fluid ($HgCl_2$ saturated in water 2 volumes, absolute alcohol 1 volume, with addition of a few drops of glacial acetic), and Mann's picrocorrosive formol solution. The first two fixatives may be used directly on the wet smears, or with previous exposure to osmic vapour for a short time. I find it useless, however, to fix the smears on slides; the reason being that it is impossible, or at least very difficult, to put them into the fixatives without making them dive in with one end foremost, a process which causes distortion in the wet and still unfixed elements of the smear. Satisfactory preparations can only be obtained by these methods of fixation, by making the smear on a coverslip and dropping it at once with the smear downwards into the fixative. I hold the coverslip with the fingers of my left hand, and a glass rod in my right hand; a drop of blood is placed by an assistant on the coverslip; I then smear it out immediately with the glass rod and drop it instantly into the fixative. The whole process can be done most expeditiously, and with much less risk of partial drying than when dealing with slides.

In staining trypanosomes with iron-hæmatoxylin, I find it best to let the mordant and stain act for a long time. The objects are first left in the iron-alum solution ($3\frac{1}{2}\ {}^o/_o$) over night; they are then, after brief and rapid washing with distilled water, transferred to the hæmatoxylin solution ($\frac{1}{2}\ {}^o/_o$) for at least 24 hours. The whole art of the process lies in the differentiation and extraction of the stain. My method is to put the coverslip into the iron-alum until colour is seen to be coming out; then the coverslip is dipped into tap-water to stop the extraction of the stain, and examined with moderate magnification (Zeiss Oc. 4, Obj. D). If the karyosome can be seen sharply and clearly in a trypanosome, the extraction is sufficient; if not, the coverslip is put into iron-alum again for a short time. Usually I do several smears of each sample of blood with different degrees of extraction of the stain. In most smears there are also thicker and thinner portions, and it is found that in thicker portions of the smear the stain is not so quickly extracted from the trypanosomes as in the thinner portions. Different degrees of extraction of the hæmatoxylin have their uses in showing up different points of structure. When the trypanosomes first come out of the stain they have an even, opaque black colour. I find that usually the stain is extracted first from the cytoplasm generally, then from the myonemes; next from the coarse granulations of the cytoplasm; next from the flagella and the blepharoplasts; next from the karyosome, and last of all from the kinetonucleus which appears to give up the colour at its periphery first. There are exceptions, however, to this order, for in some trypanoplasms (e. g. *T. gurneyorum*) the cytoplasmic granules retain the stain as long as the karyosome. In one of my preparations which was under-extracted, I found that the trypanosomes and trypanoplasms showed their form and the flagella very sharply, but were too opaque for internal structure; I therefore had the parasites drawn in outline with the camera lucida, then unmounted the coverslip, extracted more of the stain, and, after mounting it again, added the details of minute structure to the drawings already made.

With use of iron-hæmatoxylin in never-dried preparations suitably fixed, absolutely uniform results are obtained with regard to the nuclear structure. When two such staining methods as the Romanowsky stain and the Heidenhain stain give conflicting results in matters of nuclear detail, no one with any knowledge of cytological methods would hesitate, I think, to regard as more reliable the evidence yielded by the Heidenhain stain.

The figures illustrating this memoir were drawn with the camera lucida to a magnification of 2000 linear, with the exception of the sketches drawn from living trypanosomes (figs. 77, 94, 95). The majority of the drawings were executed by my assistant Miss Rhodes, to whom I desire to express my thanks for her skilled help in this work.

General Remarks on the Trypanosomes and Trypanoplasms of Fishes.

It would, perhaps, be more logical if the general account of these parasites came after the detailed descriptions of the species, since the conclusions at which I have arrived are founded on the data which are set forth in the special descriptions. But to many the general summary of results that is given here will be of greater interest than the, perhaps, rather wearisome special details, which therefore I relegate to second place, for purposes of reference for those specially interested.

From a study of the trypanosomes of fishes in the fresh, living condition in the blood, I have come to the conclusion that there are two types of movement: a conclusion which may perhaps be true of trypanosomes universally. Sometimes they may be seen to move in a definite direction; at other times they are seen to be twisting about in one spot without moving from it. I term these two types of movement travelling and wriggling respectively. They travel usually with the flagellum forwards; in the trypanosome of the eel I have observed progression of this type only. Some trypanosomes, however, can be seen to travel occasionally with the flagellum directed backwards; progression in this manner appears to me to occur chiefly when the trypanosome is forcing its way through corpuscles, and never when its path is unobstructed. In either case, however, progression appears to be effected chiefly by flexions of the whole body and by rippling movements of the undulating membrane, and scarcely at all by means of the free flagellum.

In wriggling movements the trypanosome simply twists over and over in S-like curves in one spot, and often appears as if tied in a knot; this is what I understand Laveran and Mesnil to mean by the expression "pelotonné." The object of these movements is probably simply to increase the animal's power of absorbing nutriment &c. from the surrounding medium, by incessantly changing the surface of contact between the body and the blood-plasma, just as a *Tubifex* in the mud wriggles incessantly in order to bring the surface of the body into contact with fresh water and oxygen. I think it highly probable that when these parasites are being carried round passively in the circulation they perform wriggling movements only, having no occasion to travel. On the slide, however, they are seen sometimes to travel, sometimes to wriggle; but when they become moribund they only wriggle.

Trypanoplasma, so far as I have observed, travels always with the anterior free flagellum forwards; I have never seen it go with the posterior flagellum, that is to say the flagellum which runs along the edge of the undulating membrane, directed forwards. If the kinetonuclear extremities of *Trypanosoma* and *Trypanoplasma* are to be considered homologous, then the direction in which the former usually travels is the opposite to that in which the latter invariably progresses. I have not observed in *Trypanoplasma* anything comparable to the wriggling movements of *Trypanosoma*; the former genus has the body relatively much shorter and less flexible. It can be observed frequently, however, that a *Trypanoplasma* when travelling in a certain direction will quite suddenly bend over on itself and travel in a direction more or less the opposite to that which it took formerly.

A remarkable feature of some trypanosomes and trypanoplasms of fish is the great disparity in size between different individuals in the same blood. This point has already been noticed by previous observers with regard to the trypanosomes of the pike and the eel, both of which have been divided, each into two varieties, distinguished as var. *parva* and var. *magna* respectively. In the five fish-trypanosomes examined by me, I find the state of things different in different species. In the trypanosomes of the bream and the tench, which are perhaps one and the same species (figs. 27–32), I could not find any variation in size sufficiently well marked to be characterized as true dimorphism; but my material of these two forms is not so abundant as in other cases. In the trypanosome of the pike (*T. remaki*, figs. 20–26) I found the well-marked dimorphism described by Laveran and Mesnil, and though the two forms *parva* and *magna* each vary slightly in size and other characters, they were nevertheless easily distinguished, and no forms could be found transitional between them. In the trypanosome of the perch (*T. percae*, figs. 8–14) I found three principal types—small slender forms, large stout forms, and intermediate forms. The small forms and the intermediate forms are connected by transitions (figs. 10, 11), but the stout forms stand rather apart, owing to the shortness of the free flagellum (figs. 13, 14). Finally in the eel, I found every possible gradation between the smallest and the largest forms (figs. 1–7); there is a very great difference in size between the two extremes, but the absence of a dividing line between them makes it difficult, if not impossible, to distinguish the varieties *parva* and *magna*.

The significance of this dimorphism or polymorphism is not clear, and must be explained from the life-history. Two possible explanations present themselves: first, that the difference between small and large forms is one of growth and development; secondly, that it is a manifestation of sexual differences, small male and large female forms being differentiated from an indifferent or intermediate form. The trypanosomes of the pike and the perch rather favour the sexual hypothesis, but the state of affairs in the eel-trypanosome strongly suggests stages of growth merely. Certain facts that I have observed in the perch have awakened in me the suspicion that these fish-trypanosomes have some form of multiplication in the internal organs of the fish, and that fission of the type familiar in other trypanosomes perhaps only occurs after a new infection, just as in *Trypanosoma lewisi* fission is only found in the first week or ten days after inoculation. Fission has very seldom been seen in fish-trypanosomes; so far as I am aware, it has only been seen in *T. remaki* immediately after inoculation into a pike (Laveran and Mesnil) and in *T. granulosum* in cultures *in vitro* (Lebailly, França). The absence of fission-stages in the blood of fish infected naturally is very striking. The subject is one requiring renewed investigation.

In the trypanoplasms of the tench (figs. 40–43) and pike (figs. 33–37, 56) I have also observed smaller and larger forms, sharply distinct not only in size but even in nuclear structure; the large forms are much less common than the smaller "ordinary" forms, and both types are divisible into two categories by differences in the nuclear apparatus, especially the kinetonucleus. According to Keysselitz the large forms are gametes destined to conjugate in the intestine of the leech; those with larger kinetonuclei are regarded by him as gametes of male character, while the forms with smaller kinetonuclei are regarded as female. In one of the ordinary forms of the trypanoplasm of the pike I have seen the only example that has come under my notice, of what is apparently nuclear division (fig. 62).

It is a moot point, how far different species of trypanosomes can be distinguished by morphological characters. As I have already said, the trypanosomes of the five species of fish studied by me belong, in my opinion, to four species, namely (1) the trypanosomes of the bream and tench, (2) of the pike, (3) of the perch, (4) of the eel. Each of these four putative species can be easily distinguished from the other three by its structural characters, considered as a whole; I need only refer to my figures. There is, however, always the possibility to be borne in mind that one and the same species of trypanosome, when inoculated into distinct species of fish, might vary in character in response to differences in the environment; this is a point on which experimental evidence is needed. The trypanoplasms of the tench, bream, and pike seem to me also to constitute three well-characterized morphological species, each quite distinct from the only specimen of *T. borreli* of the rudd that I have seen.

Considering the great variability in size of these parasites, it is remarkable that so many authors should base their specific descriptions so largely on measurements. A naturalist, for instance, of the attainments and experience of my friend M. Brumpt (to whom I owe my first lesson in the art of staining trypanosomes in blood-films) can hardly have thought such measurements to be of any real validity as specific characters.

In considering the minute structure of these parasites we may begin with the nuclear apparatus. As I have stated above, the use of iron-haematoxylin as a stain permits of a uniform interpretation of the appearances; and I think it is a very important point, that it is possible to give in general terms a description of the nuclear apparatus which will apply to trypanosomes and trypanoplasms alike. In both cases we find a kinetonucleus and a trophonucleus, the first named being relatively very large in the trypanoplasms, small in the trypanosomes. Near the kinetonucleus are found the minute blepharoplasts or basal granules of the flagella, two in *Trypanoplasma*, one in *Trypanosoma**.

The kinetonucleus in both cases appears as a dense body stained a uniform deep black after iron-hæmatoxylin. I have not been able to detect any structure in it. When the stain is over-extracted it appears to come first out of the periphery of the nucleus and last of all out of the central part, but this appears to me to be merely the expression of the dense texture of the kinetonucleus, and not to indicate any structural difference between central and peripheral regions. After the Romanowsky stain the kinetonucleus appears very much larger than it does after iron-hæmatoxylin; it may be four or five times as large (compare figs. 33–36 and 57–64; 40–43 and 66–72; 1–7 and 78–85, &c.). As I have stated above, I believe this result to be due to the fact that the stain forms a deposit round it. With iron-hæmatoxylin the size of the kinetonucleus is quite uniform if the stain be not over-extracted, in which case also the true size is indicated by a clear space surrounding the central portion stained black.

The blepharoplasts appear usually as very minute dots, scarcely thicker than the flagellum, after iron-hæmatoxylin. There appears to be a band of fibril connecting the kinetonucleus and the blepharoplasts, which is shown by iron-hæmatoxylin, at a certain degree of extraction (fig. 98); it stains much less deeply than the kinetonucleus. After the Romanowsky stain the blepharoplasts often appear much larger and may be conspicuous; hence this stain is useful for demonstrating their existence, although it does not exhibit them in their true proportions. The flagella also share in the general enlargement after the Romanowsky stain, and appear very much thicker than they do after iron-hæmatoxylin, which shows them as very delicate filaments; they give up the stain more readily than do the true chromatic structures.

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* I adhere, in my descriptions of the structure of these parasites, to the terminology of the organs suggested by me in the 'Quarterly Journal of Microscopical Science,' lii. pp. 171–174, text-fig. A; with the difference that I employ the term "karyosome" for the intranuclear body there termed "centrosome." To judge by its behaviour during nuclear division, the karyosome of a trypanosome probably contains a true centrosome or "centriole."

The trophonucleus is very different in its structure from the kinetonucleus. After iron-hæmatoxylin (Plates IV. and V.) it appears as a clear space, oval or round, limited by a faint but quite definite membrane, which, when carefully examined, appears granular and uneven in thickness, and is probably composed of granules of chromatin connected together; it is therefore not a true nuclear membrane, in the sense in which the term is used for Metazoan nuclei. The trophonuclear membrane is much less distinct in *Trypanoplasma* than in *Trypanosoma*; a difference perhaps largely due to its being obscured in the former genus by the numerous and deeply staining cytoplasmic granulations. In the space enclosed by the nuclear membrane lie one or more karyosomes, rounded bodies often very large, and staining very deeply with iron-hæmatoxylin. The karyosomes appear to me to be simply masses of chromatin; they retain the stain very tenaciously. Typically there is a single karyosome placed more or less centrally, but their size, number, and arrangement vary greatly, as will be seen by reference to the figures and special descriptions of the species. In the space between the karyosome or karyosomes and the nuclear membrane there are found minute chromatin granules, often disposed so as to leave a clear space round the karyosome; the granules themselves are often so minute as to be scarcely visible.

Where, it may be asked, are the definite chromosomes, eight in number, surrounding the karyosome, so often described by Schaudinn, Léger, Keysselitz and others? Where indeed! I can but describe what I have seen, and I have never seen, after iron-hæmatoxylin, more than I have described above. Possibly the Schaudinnian chromosomes make their appearance at certain stages of the development or phases of the life-history. I can only say that they have never revealed themselves to me in any of my preparations. After the Romanowsky stain, it is true, a great variety of appearances can be seen, impossible to interpret in a uniform manner: sometimes the whole trophonucleus appears an even red mass, sometimes it shows coarse granulations disposed in various ways; in all cases its great difference in size, structure, and appearance from what is seen in the iron-hæmatoxylin preparations is quite bewildering. I have put forward above, in the section dealing with technique, what I believe to be the explanation of this. I hope to discuss the whole question much more fully in dealing with *Trypanosoma lewisi* elsewhere.

Lühe has given * two figures of the trypanoplasm of the carp stained with iron-hæmatoxylin. He figures the trophonucleus as a simple black rounded mass. I am of opinion that what he has figured is simply the karyosome, and that the peripheral portion of the nucleus is not shown. The nuclear space and membrane are often not at all distinct, as I have said above, and I have often seen the trophonucleus appearing just as Lühe has figured it.

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* In Mense's 'Handbuch der Tropenkrankheiten,' iii. p. 83, fig. 5.

In the cytoplasm there are commonly numerous coarse granulations, which stain like chromatin after Romanowsky, but I feel strong doubts as to their being chromidial in nature. After iron-hæmatoxylin I found that the granules gave up the stain much more readily than the true chromatic structures, in the trypanosomes; but in the trypanoplasm of the pike they held the stain very tenaciously. Their true nature could only be determined by the development.

In the trypanosomes of the perch (figs. 96, 97) and eel (fig. 84) I was able to obtain preparations showing the myonemes after the iron-hæmatoxylin stain. I have not been able to see myonemes in any trypanoplasm. I am convinced that to see the myonemes it is necessary to get just the right degree of extraction of the stain; a shade less extraction, and the myonemes are obscured by the darkness of the cytoplasm; a shade more, and the myonemes give up the stain. The darkly staining, opaque cytoplasm of the trypanoplasms probably makes it much more difficult to render visible the myonemes than in the case of the trypanosomes. From my preparations the myonemes appear to be about eight in number in the trypanosome of the eel, but more than that in the trypanosome of the perch. I have never been able to make them out with certainty after the Romanowsky stain.

With regard to the question of the transmission of the trypanosomes and trypanoplasms of fishes, on which much light has been thrown by Brumpt and Keysselitz, I have only a few negative results to record. I found leeches very seldom on the fish caught by me; only once on a rudd, and once on a perch, a *Piscicola* was found attached. On the other hand almost every fish, of whatever species, had *Argulus* attached to it when caught; sometimes there would be five or six *Arguli* on one fish. *Argulus* is stated in all the memoirs and text-books dealing with it to feed on the blood of fishes; it is an active swimmer, and readily leaves one fish and attaches itself to another. It seemed to me therefore that *Argulus* was a creature admirably suited by its habits to disseminate blood-parasites, either by the direct mechanical method, or with a cycle of development. Moreover, *Argulus* is beautifully transparent, and everything in its interior can be seen in the living animal under the microscope; it is easy to focus its blood-corpuscles flowing through the heart and circulating in all parts of its body-cavity, or to see all the contents of its digestive tract, without injuring the beast in any way. It would be quite an ideal form in which to study the development and transmission of hæmoflagellates. I took numerous *Arguli*, kept them hungry for a day or two, and then put them into bell-jar aquaria containing various fishes. As a rule the *Arguli* attached themselves at once to the fish very firmly. After leaving them for varying periods I removed the *Arguli* and examined them, first living and uninjured under the microscope, and afterwards dissected in salt-solution and examined fresh. Although the fishes on which the *Arguli* fed contained trypanosomes or trypanoplasms in all cases, I never found a flagellate of any sort or description in the *Arguli*, however the experiment was varied; and, moreover, I never in any case found the *Arguli* to contain blood-corpuscles. It might be supposed that an animal so transparent as *Argulus* would show the effects of a meal of blood by a change of colour visible to the naked eye; but in no case could a change of colour be discerned by any optical means whatsoever. I very much doubt, therefore, if *Argulus* feeds on blood, or at least on blood-corpuscles. Very possibly only fluid plasma or lymph can pass the very minute terminal aperture of its proboscis.

My experiments with leeches were greatly hampered by the fact, in the first place, that I can lay claim to no special knowledge of the Hirudinea, and secondly, that no general monograph of this group was accessible to me of later date than that of Moquin-Tandon (1846). It would be a great boon to those studying these parasites if a more recent monograph or handbook were in existence. It is not every student of hæmoflagellates who is at the same time, like M. Brumpt, a first-class expert on leeches. I obtained a few specimens of *Piscicola*, but found nothing in them of flagellate nature. I put a *Piscicola* in an aquarium in company with a perch; the leech took no notice of the fish, nor the fish of the leech. After some days I removed the leech, dissected it, and found nothing in it. I also caught in the Broad some other leeches, which I could not identify accurately, and put them in with perch; in all cases the leeches vanished completely, and were apparently eaten by the fish. *Voilà tout!*

I can therefore make no positive additions to the transmission-question, but I hope to return again to this point when opportunity offers itself.

Description of the Species.

1. Trypanosoma percæ Brumpt. (Plate I. figs. 8–15; Plate V. figs. 94–104.)

Brumpt (CR. Soc. Biol. lx. 1906, p. 161) describes this species as follows:—" Dans le sang de la Perche (*Perca fluviatilis*). Ce parasite mesure 57μ de longueur totale, dont 16μ pour le flagelle, et 3μ de large. Le blépharoplaste est à 1μ·5 de l'extrémité postérieure. Le noyau se trouve à égale distance de la racine du flagelle et de la partie postérieure."

I identify the trypanosomes found by me in the Perch as *T. percæ* Brumpt solely on the ground of occurrence, since Brumpt's description might apply to almost any trypanosome, and fails to note any of the peculiarities of the trypanosome of the perch, or to take into account its variations of size and structure.

Trypanosomes were found more or less abundantly in almost all the perch examined by me. In a few of these fish none was seen, or they were found only after more prolonged searching; as a rule, however, they were found at once and were present in considerable numbers. If a fish was not well infected, I did not trouble to make smears of it or to examine it further. Hence the few cases in which I did not find trypanosomes were probably cases in which careful search would have revealed the presence of the parasites in scanty numbers. I doubt if any of the perch were really entirely free from them. From the perch I examined, I gained the impression that the trypanosomes were rather more abundant in small or medium-sized fishes than in those of the largest size. I did not find trypanoplasms in any of the perch examined *.

Examination of the blood freshly drawn from the perch showed that the trypanosomes differed considerably in size. They may be divided, speaking generally, into large and small forms. The large forms were much the most abundant; the small forms were very scarce. The large forms when seen living (figs. 94, 95) appeared stout and sluggish, as a rule of considerable size, but showing marked variations in this respect, some being smaller and more active. They wriggle incessantly but do not travel much. The body is spindle-shaped; one end, which bears the flagellum, is greatly attenuated and sharply pointed; the other is also drawn out, but is much less attenuated and appears to end more bluntly. At the blunt end a distinct, very refringent dot could always be seen, doubtless representing the kinetonucleus; it appeared light at a high focus, black at a low focus. Sometimes two small dots could be seen (fig. 94), in which case the second was probably the blepharoplast. The nucleus could be seen distinctly as a rounded clear space in which the karyosome appeared as a darker spot, not very refringent. A short way behind the nucleus there was seen in some specimens a distinct dot or grain, much more refringent than the karyosome, and apparently lodged in a clear space or vacuole (fig. 95); this body was not always seen. The undulating membrane and flagellum could be clearly distinguished, especially when the parasites were moribund and becoming slower in their movements, after being under observation for some hours.

The trypanosomes show a great tendency in the living state to twist and roll themselves up, in a way that calls to mind a snake of stout build, such as a python. Their movements were of two kinds, which have been distinguished above as wriggling and travelling movements respectively. When wriggling they simply twist over and over, throwing themselves into **S**-like curves, without changing their place. When travelling the body is stretched out, with the undulating membrane rippling actively, the undulations commencing at the flagellar extremity and passing backwards down the body. They travel usually with the flagellum forwards and the body straightened out; the movement is effected apparently, chiefly by the undulating membrane; the blunter posterior prolongation of the body meanwhile performs curling or wagging movements which appear to be of a passive kind, the result of the movements of the undulating membrane. I saw a trypanosome, travelling in this manner on a slide under a coverslip, approach and go straight through a closely packed mass of blood-corpuscles, flagellum foremost and body straight. The same trypanosome was seen also to reverse its movement and move with flagellum directed backwards; the blunter extremity of the body, now foremost, performed movements which appeared to be active in nature, and to assist the body in penetrating forwards. This was observed also in other cases. Trypanosomes which were travelling actively were observed to come to a standstill and perform simply wriggling movements; conversely, trypanosomes which have been wriggling for some time may start off and begin travelling.

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* Keysselitz (Arch. Protist. vii. 1906, pp. 2, 3) records the occurrence of both trypanosomes and trypanoplasms in *Perca fluviatilis*.

The smaller forms were very similar to the large forms in their general appearance and the character of their movements, and only differed in their small size, pronounced slenderness, and much greater activity and rapidity of movement.

The trypanosome of the perch does not seem to possess the extraordinary vitality of *T. granulosum* of the eel. I found that the trypanosomes did not live more than a few hours *in vitro*. I made an observation, however, relating to their vital powers which perhaps is not without interest. From a perch which was very well infected, and from which I made a number of smears (fig. 11 was taken from this fish) I took three drops of blood; one drop was simply placed on a clean slide and covered with a cover-glass; the second drop was placed on a slide and mixed with a drop of Laveran's salt-citrate solution, and then covered; the third drop was put on a slide and mixed with a drop of tap-water, and then covered. The three drops were studied from time to time. In the drop mixed with water the trypanosomes showed greatly increased activity after about an hour, travelling with great rapidity in the hæmolyzed blood; after four hours they were still very active, but not quite so active as they had been earlier. After five hours I slipped off the coverslip, smeared out the blood, dried it, fixed it with absolute alcohol, and stained it with Giemsa's stain. The trypanosomes stained well and appear perfectly normal. In the drop of blood mixed with salt-citrate solution the trypanosomes also remained active but some of them were changed in form after four hours, the body becoming pear-shaped, with most of its substance aggregated at the hinder end. In some cases the trypanosomes appeared as if anchored by the hinder end; the anterior end of the body lashed round in all directions, but seemed unable to move the inert posterior mass. After about five hours I slipped off this coverslip also and made a preparation of the blood, but it only shows trypanosomes very badly preserved and stained, and apparently quite degenerate in structure. In the control drop of pure blood the trypanosomes were all dead or moribund after four hours, and I did not proceed further with it. If any conclusion can be drawn from these experiments, it is that the trypanosomes live, and remain normal, longer when the blood *in vitro* is mixed with water than when it is mixed with salt-citrate solution or left pure; a result which I certainly did not expect.

In preparations the trypanosomes of the perch show a continuous gradation of sizes from the smallest to the largest (figs. 8–14); the larger forms being, however, by far the commonest. The best means of classifying them is by the free flagellum, which in the large stout forms is very short (figs. 13, 14), but in the medium-sized (fig. 12) and small forms (fig. 8) is much longer. The cytoplasm stains a very deep blue with the Giemsa stain, so deeply in fact that it is very difficult to obtain satisfactory preparations of the stout forms; they appear often as bluish opaque masses in which the intensity of the stain obscures all details of structure. In the same preparations, on the other hand, more slender forms may be found perfectly stained. The cytoplasm in the largest forms usually appears blotchy, with lighter and darker parts, often with tiny vacuole-like spaces, not very sharply limited. Any of the forms may contain red-staining granules to a greater or less extent, sometimes very numerous, sometimes absent altogether. The granules in question are of fair size and more or less irregular in form. In one specimen I saw them frequently in pairs, and sometimes rod-shaped, suggesting division (fig. 104); and the idea occurred to me that they might perhaps be intrusive organisms of the nature of Bacteria. In never-dried preparations stained with iron-hæmatoxylin the cytoplasm appears more or less evenly and coarsely granular, according to the degree of extraction of the stain (figs. 96–99). The above-mentioned granules are not brought out by this stain. I see, therefore, no reason for regarding them as chromidia.

The nucleus appears, in the smears stained by Giemsa's method (figs. 8–14), as a red patch, often obscured and difficult to make out clearly in the large stout forms. It varies in size with the dimensions of the trypanosomes, and is much larger in the large forms. With the Romanowsky stain the details of nuclear structure appear to vary greatly; sometimes a distinct sharply limited karyosome, stained a deeper red than the rest of the nucleus, can be made out (fig. 13), sometimes not. It would appear as if the method of drying had the frequent result of distorting or breaking up the fluid or plastic karyosome, thus producing different appearances in different cases. On the other hand, in never-dried smears stained with Heidenhain's iron-hæmatoxylin, the structure of the nucleus appears quite uniform in all its principal features (figs. 97–102), in all cases, and can be described in quite general terms. It is seen that the nucleus is an ovoid or nearly spherical space limited by a delicate membrane. In the interior is a deeply staining karyosome, which may be spherical, ovoid, pear-shaped, or even dumbbell-shaped. The karyosome is always large, and sometimes so large as to nearly fill up the entire nuclear cavity and appears to be immediately surrounded by a clear space. The remainder of the nucleus is occupied by a faintly granular material, which, owing to the excentric position of the karyosome, forms usually a crescentic area on one side of the nucleus. In this area coarser dots of chromatin can be made out, especially in the neighbourhood of the nuclear membrane, which is probably composed also of chromatin. No details of structure could be seen in the karyosome itself.

The foregoing statements apply to the nuclear structure of the large or medium-sized forms, since I was not able, unfortunately, to find any of the small forms, always rare, in my preparations stained with iron-hæmatoxylin.

The kinetonucleus appears as a rounded or ovoid mass in preparations stained by the Romanowsky method. It is larger, both absolutely and relatively, in the very small trypanosomes than in the large. In preparations stained with iron-hæmatoxylin it appears either rod-shaped or rounded in form, but in either case very much smaller in size than it appears when stained by the Romanowsky method. If the hæmatoxylin be not much extracted, the kinetonucleus is often difficult to distinguish from the blepharoplast, the two together appearing to form a single mass of triangular or irregular outline. If, on the other hand, the extraction of the hæmatoxylin be carried too far, the blepharoplast and flagellum become completely decolorized and the kinetonucleus appears as a very sharply defined and deeply stained body, in which no details of structure could be distinguished.

The flagellum arises from a distinct blepharoplast, which can almost always be seen clearly, and usually lies close beside the kinetonucleus. In some cases, however, the blepharoplast and kinetonucleus are separated by a considerable interval, and then appear to be connected by an ill-defined band of material staining more lightly (fig. 98). In the living condition also, as stated above, I observed in some cases two separate grains, which apparently represented the kinetonucleus and blepharoplast.

The flagellum arises direct from the blepharoplast and runs along the edge of the undulating membrane in the usual way. As already stated, the free flagellum is short in the large stout forms, long in those of medium or small size. It is worthy of note that in the smaller forms the free flagellum ends distally in a distinct grain or dot. The undulating membrane is shallow, with many small pleats as a rule, in the stout forms with short free flagellum (fig. 13); but in all forms with a long free flagellum, and especially in the smallest forms, the undulating membrane is very deep, and stands out far from the body, with fewer and larger pleats (figs. 8–12). It generally appears clear, but in some cases the granules of the cytoplasm can be seen extending up into it, forming a distinct contour-line close under, but quite separate from, the flagellum (fig. 12).

In one of my preparations, which had been fixed first with osmic vapour and then with Schaudinn's fluid, without drying, and stained with iron-hæmatoxylin, I found the trypanosomes showing distinctly striations which are doubtless to be explained as myoneme-filaments (figs. 96, 97). They are to be made out on both surfaces of the body running spirally, and hence appear to cross in the drawing, but in the object they are seen at different foci on the two surfaces. In one trypanosome they appear to run in couples (fig. 96). In another, which was much bent up and probably contracted, the myonemes can be seen on one surface running to a very convex edge, where they appear in optical section as distinct grains, and from this point they can be traced again on the other surface (fig. 97). The exact number was difficult to make out; fig. 96 indicates that there are in all 8, or 4 couples, but in fig. 97 there appear to be more than this. To these myonemes may be referred the active wriggling movements of the trypanosomes.

In some of my preparations stained with Giemsa's stain I found very broad forms of the trypanosome. I am convinced that these forms are simply stout forms of the trypanosome deformed and flattened out by drying. In slides fixed with osmic vapour before drying I find them only at the edge, or in very thin parts of the film, that is to say in places where it is difficult to avoid a slight amount of drying taking place. Two other points are to be noted in favour of this conclusion; one is that the very broad forms are not so opaque as the stout forms which do not show any flattening; the other is that the nucleus is more or less considerably elongated in the transverse direction, having evidently shared in the increase of breadth produced by the flattening.

In one preparation, fixed wet with osmic vapour and stained by Giemsa's method, I found a trypanosome apparently encysted (fig. 15). No trace of a flagellum was to be seen, but the body is rounded off and surrounded by an envelope staining a faint bluish tinge. The shape of the nucleus indicates perhaps that a slight amount of flattening has taken place. No other similar stage was found.

In preparations from a perch which showed abundant trypanosomes in its blood, I found, on two separate slides, two bodies resembling hæmogregarines (fig. 16); they were free in the blood-plasma and strongly resembled the free vermicules of these parasites. It is well known that hæmogregarines occur commonly in marine fishes, but in freshwater fishes they have only been found in the eel. I searched long, but in vain, for intracorpuscular stages similar to the vermicules; I found, however, in the large uninucleate* leucocytes, bodies which I took at first to be parasites: rounded bodies staining a faint pink (after Giemsa's stain), with a central darker grain or two such grains (figs. 17–19). I could not find, however, anything in the least transitional to the vermicule-like bodies, and I do not think now that the bodies in the leucocytes are of parasitic nature. In the corresponding leucocytes of the tench (figs. 45–48) there are to be found commonly, but not invariably, round pink-staining bodies, sometimes a single one of variable size, sometimes two, three, or four such; they are clearly vacuoles containing some substance which has stained, probably, with the eosin of the Giemsa stain, and I think it very probable that the bodies in the leucocytes of the perch are of a similar nature.

If the vermicules, however, are not stages of a hæmogregarine, in what light are we to regard them? Since they were found in the same blood as the encysted trypanosome already described (fig. 15), the idea occurred to me that perhaps the stout forms of *Trypanosoma percæ* might encyst in the internal organs and undergo multiplication to form the vermicule-like bodies; these in their turn might acquire flagella and so give rise to the smallest forms of the trypanosome, which by growth into the large forms would complete a cycle of multiplication in the fish. The rarity of fission-stages of the trypanosomes of fish is remarkable; I have never seen a fish-trypanosome in division, but Laveran and Mesnil have described fission of *T. remaki* in two pike infected artificially, and Lebailly and França have, as stated elsewhere, described fission in *T. granulosum* from cultures *in vitro*. It is therefore quite possible that fission may be a process restricted to certain parts of the life-cycle, and that the usual mode of multiplication in fish-trypanosomes may be such as I have indicated above. In *Trypanosoma lewisi*, for example, fission is only found during the first week or so after inoculation; there is then no further multiplication, the trypanosomes being all of one size and type. On the other hand, fish-trypanosomes usually exhibit marked variations in size which are very suggestive of growth from the smallest to the largest forms. I desire to make this suggestion cautiously, as the data on which it is founded are obviously quite inadequate to establish it. I may point out, however, that the blood in which the encysted trypanosome and the vermicule-like bodies were found, was taken from the heart of the fish with a capillary glass tube, and it is quite possible that the tube in passing through the walls of the heart may have taken up bodies which were not free in the general circulation, but contained in the wall of the heart itself.

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* Commonly termed "mononuclear"; a barbarous etymological compound. The adjective "nuclear" means "of or relating to the nucleus"; not "possessing a nucleus."

2. TRYPANOSOMA GRANULOSUM Lav. & Mesn. (Plate I. figs. 1–7; Plate V. figs. 78–93.)

The trypanosome of the Eel has been seen by many observers, and there is no other fish-trypanosome which has been the object of so many memoirs. According to Laveran and Mesnil, the first description of this parasite was by Sabrazès and Muratet, but the earlier work of these authors is not accessible to me. The earliest memoir on the subject with which I am acquainted is that of Laveran and Mesnil themselves (1902), in which they figure and describe the parasite and name it *T. granulosum*. Lebailly (1906) gave a detailed description, with two figures, of this trypanosome and distinguished two varieties, *magna* and *parva*. In the same year Brumpt described the transmission of the trypanosome of the eel by the leech *Hemiclepsis*, and the development that the parasite goes through in this leech. Finally, França (1907) has devoted a memoir to this trypanosome.

Since so much work has been done on the trypanosome of the eel, I did not pay much attention to it, not expecting to be able to add much to our knowledge on this subject. I obtained four eels caught in the Broad and examined one of them, which I found to contain trypanosomes most abundantly, more so than any fish I have yet examined. I made a number of smears and preparations from this eel and then sent all four eels to the kitchen, to be prepared for my next meal; it was not found that infection with trypanosomes impaired appreciably their gastronomic properties. I much regretted afterwards that I did not examine the other three eels to see if they were equally well infected.

Sabrazès & Muratet (1904), Lebailly (1906), and França (1907), have all drawn attention to the extraordinary vitality of this trypanosome, and the fact that if a drop of blood containing them be sealed up on a slide under a coverslip, the trypanosomes will live for several days and multiply. I have not imitated these experiments, but I had a drop of blood under a coverslip, not sealed up in any way, in which the trypanosomes were moving actively after 24 hours, less actively after 48 hours, and feebly after 72 hours.

I spent some time watching the movements of this trypanosome, which are extraordinarily snake-like. As in the case of *T. percæ*, the movements are of two kinds, conveniently distinguished as wriggling and travelling. They may wriggle for a long time without changing place to any considerable extent, and then they may suddenly start travelling. When they become weaker, after 24 hours under a coverslip, they only wriggle and do not travel, and as they become moribund the wriggling movements become weaker and weaker, till on the third day they are very feeble. When wriggling they twist over and over in S-like curves, appearing at the first glance like a writhing knot. When travelling, I observed them always progressing actively in a definite direction, flagellum forwards, the body twisting from side to side in even curves like a snake. They frequently stop suddenly, and wriggle actively in one place like an excited earthworm, and then start off again in a new direction. I never saw them travel with the flagellum directed backwards. The undulating membrane is very distinctly seen, and shows beautiful rippling movements. Progression appears to me to be effected chiefly by twists and turns of the body, aided doubtless by the undulating membrane; the flagellum has perhaps chiefly a tactile or guiding function. The kinetonucleus is very distinct in the living condition as a refringent granule a short way from the hinder end. The trophonucleus is more difficult to see during life.

Although my preparations were made only with the object of adding to my collection, they show some points of interest, which I will state briefly.

In the first place my preparations show trypanosomes of all sizes from very small to very large (figs. 1–7). The contrast between the two extremes of the series is surprisingly great, but all possible transitions from one extreme to the other are to be met with. Nor can I find any definite structural features to separate sharply the large and small forms, as can often be done in trypanosomes of other species. The large forms are very granular, while in the smallest granules are absent or scarce, but this feature shows both variations and transitions; the large forms have more pleats in the undulating membrane than the smaller, but the pleats are about the same depth and simply increase in number with the length of the trypanosome. The free flagellum bears about the same proportion, as regards length, to the rest of the body in both large and small forms, though in some large forms it is perhaps relatively shorter. The differences in the nucleus, to which I shall refer presently, are also differences due to a gradually increasing complexity of structure. I cannot therefore find characters to separate sharply the varieties *magna* and *parva* of Lebailly.

A very marked feature of this species, when stained by the Romanowsky method, is the occurrence of numerous granules in the cytoplasm, a peculiarity to which it owes its name. The granules stain purple or blue with Giemsa's stain. In preparations stained with iron-hæmatoxylin, the granules are only to be seen when the stain is not sufficiently extracted (fig. 83); when the stain is most satisfactory and the flagellum and nuclei are sharp and distinct, the characteristic granulations are not to be seen. One of my coverslip-smears is rather uneven and thicker in some parts than in others. In the thicker portions the stain is less extracted than in the thinner parts of the smear, as commonly happens, and hence different degrees of extraction can be found in the preparation. Only a very few trypanosomes show the granules stained and standing out sharply; with too slight extraction of the stain the granulations are obscured by the darkness of the cytoplasm. If the stain is very much over-extracted, as in one of my preparations, the colour comes out of everything except the kinetonucleus; when the extraction has not gone quite so far, the karyosome of the trophonucleus also retains the black colour. The granulations of the cytoplasm are not, in my opinion, to be regarded as chromidia, since they give up the stain very readily, while the true chromatic structures retain it very tenaciously.

França has drawn attention to the peculiar structure of the nucleus in the variety *magna* of this trypanosome. He writes:— "Le noyau ..... s'éloigne par sa structure de celui de presque tous les Trypanosomes. Au sein d'une substance incolore on voit la chromatine formant d'ordinaire deux parties bien distinctes; un grand bloc ..... d'un rouge vif, situé dans la partie du noyau la plus rapprochée du blepharoplaste; et un grand nombre de granulations petites et régulières, ou un reticulum chromatique, vers le coté du flagelle. Dans quelques parasites, plus rares, on ne voit pas le grand bloc de chromatine et alors il existe dans la substance nucléaire incolore une série de granulations disposées de façon à former un cordon plus au moins sinueux." "Au contraire de ce qui existe dans le noyau des Trypanosomes de la var. *magna*, dans ceux de la var. *parva* il n'y a pas une distinction nette entre les portions chromatique et achromatique."

França's observations appear to have been made on trypanosomes stained by the Romanowsky method, and my preparations coloured with Giemsa's stain confirm his statements. But I do not believe it is possible to get a coherent or intelligible idea of the structure of the nucleus with this method of staining. My preparations stained with iron-hæmatoxylin show appearances quite different from those seen after use of the Romanowsky stain, and at the same time permit of a uniform interpretation of the structural details. The nucleus appears very much smaller and more compact after iron-hæmatoxylin than after the Romanowsky stain; and while it is possible that there is a certain amount of shrinkage by the former method, I think there is certainly a considerable amount of artificial expansion and deformation consequent on the process of drying by the ordinary method of applying the Romanowsky stain.

In never-dried preparations stained with iron-hæmatoxylin, the smallest trypanosomes (figs. 78, 79) show the nucleus as a clear oval space with a distinct limiting membrane, containing a sharply defined karyosome of elongate-oval form. The karyosome is far from filling up the entire nuclear space, which appears clear or shows very minute granulations. A nucleus of this type of structure may be found even in trypanosomes of large size (fig. 87), but the karyosome in such specimens is considerably larger and fills up nearly the whole nuclear cavity. As a rule, however, in trypanosomes only slightly larger than the smallest that can be found (figs. 80, 81), the karyosome is seen to have budded off from one extremity, usually from that furthest from the kinetonucleus, a smaller part. This is the type of nucleus most commonly found, one in which there are two karyosomes, either subequal, or markedly unequal, in size (figs. 85, 86, 93). But in many of the large forms the karyosome may be further broken up into three or even four parts, usually differing greatly in size (figs. 89–92). Thus, with endless variations in detail, a similar type of nuclear structure occurs with monotonous regularity in all the preparations stained with iron-hæmatoxylin; always an oval space containing one or more deeply stained karyosomes. What may be the significance of the disruption of the karyosome I am unable to say.

The kinetonucleus appears after iron-hæmatoxylin as a rounded or rod-shaped body of minute size. After staining by the Romanowsky method it appears very much larger and of various shapes which are seen in my figures. Near it a distinct blepharoplast can be made out, from which the flagellum arises directly. The free flagellum is usually of considerable length. The undulating membrane does not stand out so boldly from the body as in some species of fish-trypanosomes. In one preparation I saw distinctly a line under the flagellum (fig. 82), indicating doubtless the limit of the extension of the endoplasm into the undulating membrane. Only in one specimen was I able to make out myonemes (fig. 84). They appeared as delicate lines, about three being visible on the side of the body, a number which may correspond to the total of eight alleged to be typical of trypanosomes.

3. Trypanosoma remaki Lav. & Mesn. (Plate II. figs. 20–26.)

The trypanosome of the Pike has been described by Laveran and Mesnil (Arch. Protist. i. 1902, pp. 482–486, figs. 1–9). I have very little to add to their description of this parasite, which I found in four out of the five pike examined by me at Sutton Broad. Laveran and Mesnil have noted that this parasite has a wide distribution in Europe, its occurrence having been noted by several naturalists.

Laveran and Mesnil noted two forms which they distinguished as var. *parva* and var. *magna*. I found these varieties very distinctly marked in the pike examined, and without any transitional forms connecting the two extreme types. Both var. *parva* and var. *magna* are subject to slight variations in size, but I found no difficulty whatever in distinguishing the two types in my preparations, whether fixed by osmic vapour or by the ordinary method of drying. The var. *parva* (figs. 20–22, 25) is clearer, with but few coarse granulations, its undulating membrane is shallow and does not stand out sharply from the body, and its free flagellum is longer, relatively and absolutely, than that of var. *magna* (figs. 23, 24, 26), which contrasts with it not only in greater dimensions, but also in its opaque cytoplasm full of coarse granules staining reddish with the Giemsa stain, and in its very prominent undulating membrane. The kinetonucleus also seems to be slightly larger in var *parva* than in var. *magna*; but I am not able, unfortunately, to make precise statements concerning the nuclear structure, since in the smears which I stained with iron-hæmatoxylin I was unable to find any trypanosomes, though *Trypanoplasma gurneyorum* was abundant.

4. Trypanosoma tincæ Lav. & Mesn. (Plate II. figs. 27–29; Plate IV. figs. 74–76).

The trypanosome of the Tench was described and named by Laveran and Mesnil (Trypanosomes et Trypanosomiases, Paris, 1904, pp. 387, 388, fig. liii. 2 & 3). Its occurrence had been noted previously by Doflein (Die Protozoen als Parasiten etc., Jena, 1904, p. 71), and since then by Keysselitz (Arch. Protist. vii. 1906, p. 3). Doflein considered that the parasite seen by him might be identical with *Trypanosoma carassii* from *Carassius vulgaris*, but Laveran and Mesnil regarded it as a distinct and new species, and described it as follows:—

"La longueur est de 35 μ en moyenne, la largeur de $2\frac{1}{2}$ μ à 3 μ. L'extrémité postérieure est conique, peu effilée. Le centrosome, assez gros, est voisin de l'extrémité postérieure. Le noyau est situé vers la partie moyenne du corps du parasite. La membrane ondulante est large, bien plissée. La partie libre du flagelle est assez longue."

I found trypanosomes in all tench examined by me at Sutton Broad, occurring together with *Trypanoplasma keysselitzi*, and have observed nothing to prevent me identifying them with *Trypanosoma tincæ* of Laveran and Mesnil. This trypanosome appears to be very uniform in size and structure, so far as my observations extend; the two specimens figured by Laveran and Mesnil differ somewhat in size. In my never-dried preparations fixed with Schaudinn's fluid, with or without previous fixation with osmic vapour, and stained with iron-hæmatoxylin (figs. 74–76), the trypanosomes appear constantly smaller than they do in preparations fixed wet with osmic vapour, or dried before fixation, and stained with Giemsa's stain; but I attribute this difference to the effects of the method of fixation.

In osmic-fixed preparations the trypanosome shows, as Laveran and Mesnil have stated, a very broad undulating membrane, standing out well from the body, and continued into a free flagellum of moderate length (figs. 28, 29, 36); the cytoplasm is opaque in such preparations and usually shows numerous coarse granules, which stain reddish with Giemsa's stain, and by the iron-hæmatoxylin method appear black and hold the stain fast. The kinetonucleus appears large after Giemsa; but very much smaller after iron-hæmatoxylin, and by the latter method shows two types of form, rounded and rod-shaped (figs. 74–76). The blepharoplast is not easily seen in the Giemsa preparations, owing to the opacity of the cytoplasm, but in iron-hæmatoxylin preparations it appears as a sharp dot near the kinetonucleus. The trophonucleus appears after Giemsa stain as an oval patch showing no details of structure; after iron-hæmatoxylin it appears as a rounded or oval space, not very sharply limited, containing a very large round karyosome. In one preparation the karyosome appeared to fill the whole nuclear space and to be of irregular form (fig. 75); a result perhaps due to insufficient extraction of the stain.

5. Trypanosoma abramis Lav. & Mesn. (Plate II. figs. 30–32.)

The occurrence of trypanosomes in a Bream was noted by Laveran and Mesnil in 1902 (Arch. Protist. i. p. 478) and the species was named by these authors, but without any figure or description, in 1904 (Trypanosomes et Trypanosomiases, p. 388). Keysselitz also noted the occurrence of this parasite (Arch. Protist. vii. p. 3). According to Brumpt, the trypanosome of the bream goes through its development in the leech *Hemiclepsis*.

In my preparations of the blood of the bream from Sutton Broad I have found a trypanosome very sparingly; three specimens altogether, one from one fish, two from the other. The trypanosome is of large size and presents no character by which I can distinguish it morphologically from *Trypanosoma tincæ*. I consider it very probably identical with this species; in which case the name *tincæ* has priority over *abramis* by one page; but in view of the scantiness of my observations I refrain from taking the step of merging the two species into one.

6. Trypanoplasma gurneyorum, sp. n. (Plate II. figs. 33–39; Plate IV. figs. 56–65.)

The occurrence of a trypanoplasm in the Pike has only been noted, so far as I am aware, by myself (Quart. Journ. Micr. Sci. lii. p. 253, text-fig. B). I found it abundantly in all pike examined at Sutton Broad, usually occurring together with *Trypanosoma remaki* Lav. & Mesn.; in one pike, however, I found a very abundant infection with the trypanoplasm, but no trypanosomes. I propose to name this trypanoplasm *T. gurneyorum*, in honour of Messrs. Eustace and Robert Gurney, in whose laboratory this work was done.

Trypanoplasma gurneyorum occurs under two forms, which I will distinguish as "ordinary" and "large." The large forms appear to be very scarce; I have seen only two specimens so far, both in preparations from the fish mentioned above, in which only trypanoplasms were found. The ordinary form of *T. gurneyorum* (figs. 33–37) is characterized by a fairly broad undulating membrane and by the shortness of the free flagella. The anterior free flagellum is scarcely more than half the length of the body. The posterior free flagellum projects only a very short distance beyond the hinder end of the body; in some specimens it is rather longer, but it is always short by comparison with other trypanoplasms. The trophonucleus is near the middle of the body, sometimes even in the posterior half. The form of the anterior extremity of the body shows variations, which are perhaps due partly to the animal's movements, partly to deformations induced by fixation. Sometimes the anterior end appears pointed and the marginal flagellum runs backwards from its point of origin (fig. 36). On the other hand, the anterior end may be very blunt and rounded, and the marginal flagellum runs first forwards and then backwards from its point of origin (fig. 33). The second type is found in an extreme form in preparations dried before fixation (fig. 37), and is then undoubtedly largely a case of deformation; the chief mass of the body seems to flow forwards when dried, with the result of bringing the kinetonucleus and trophonucleus close together. In extreme cases the trypanoplasm becomes a shapeless mass, in which the orientation of the body is difficult to make out (figs. 38, 39). It is interesting to note that trypanoplasms deformed in this way beyond recognition occur in preparations in which the trypanosomes have their body form and structural characters preserved quite perfectly.

The large form of *Trypanoplasma gurneyorum* (fig. 56) also has the free flagella relatively short. The body cytoplasm is very dense and opaque, staining a very deep blue with Giemsa's stain, so that it is very difficult to make out the nucleus; in the specimen drawn in outline in fig. 56 I could see the kinetonucleus plainly, especially with green light, but the trophonucleus was very difficult to distinguish from the cytoplasm, and I am by no means certain that I have drawn it correctly.

The most remarkable feature of *Trypanoplasma gurneyorum* is the presence of deeply staining granules in the cytoplasm. They occur chiefly towards the hinder end of the body, but are found also, though more sparingly, up to the anterior extremity. By the Romanowsky method (figs. 33–39) they stain deeply in a colour approaching the tint of the nuclei, and more especially that of the kinetonucleus. By the iron-hæmatoxylin method they hold the stain very fast (figs. 57–65), quite as fast as do the nuclei. a point in which they contrast with the cytoplasmic granules of *Trypanosoma granulosum* and *T. percæ*. The granules are especially sharp and clear in preparations fixed with sublimate-acetic and stained with iron-hæmatoxylin (figs. 57–61). They are rounded and vary in size from small dots to coarse grains. Sometimes there are only a few granules, but as a rule they are very numerous and give this trypanoplasm a very characteristic appearance.

The nuclei of *Trypanoplasma gurneyorum* appear very different according to the stain used, Romanowsky or iron-hæmatoxylin. The trophonucleus is lodged in or near the edge of the undulating membrane, and hence is difficult to make out clearly if the undulating membrane in this part be folded over the body. It appears after the Romanowsky stain as a lightly stained patch, more or less oval in form, showing no detail or only a few irregular granulations. After iron-hæmatoxylin (figs. 57–64) it appears as a clear oval space, not very well defined, containing usually a single karyosome. In the ordinary forms the karyosome is small, often no larger than one of the coarser cytoplasmic granules, with which it may be confused very easily, so that at first sight the trypanoplasms appear to have no trophonucleus. In the only specimen of the large forms which I have seen stained by this method, the karyosome was very large and almost filled the nuclear space (fig. 65). In one specimen of the ordinary forms I found what appeared to be nuclear division (fig. 62); two karyosomes connected by a distinct curved black line, one of them in the ordinary position of the trophonucleus, the other further forwards, on a level with the kinetonucleus. In another specimen the karyosome appeared to be budding off a smaller part (fig. 59), perhaps as a preliminary to division.

The difference between the nucleus in specimens stained by the Romanowsky and the iron-hæmatoxylin methods is very striking. One sample, however, of Giemsa's stain which I used gave different results from all others, for some unexplained reason; with this sample the flagellum stained blue instead of red, and the trophonucleus appeared as a small granule (fig. 36), just as in the iron-hæmatoxylin preparations; the kinetonucleus appeared very large. Hence all the trypanoplasms stained with this sample of stain gave the impression, at first sight, of lacking a trophonucleus.

The kinetonucleus appears very large after the Romanowsky stain, much smaller after iron-hæmatoxylin, but in both cases larger, as a rule, than the trophonucleus. There are two types of form exhibited by the kinetonucleus: a more rounded or oval, broader type (figs. 57, 59), and a more elongated, band-like type (figs. 61, 64); the latter often appears to have a small piece at the anterior end constricted off from the main body (figs. 58, 64; compare Keysselitz, Arch. Protist. vii. p. 36, fig. 45). The kinetonucleus stains an even black in iron-hæmatoxylin, a deep purple after Giemsa, and shows no structural detail. Having only seen two specimens of the large form of this trypanoplasm, I am unable to say if distinct types of kinetonucleus are exhibited by it, as by the large forms of *T. keysselitzi*. In front of the kinetonucleus are two minute blepharoplasts from which the flagella arise. After the Romanowsky stain the blepharoplasts appear a fair size and are distinctly seen; after iron-hæmatoxylin, on the contrary, they are very minute and often appear as a single granule, being close together and difficult to resolve into two distinct dots. When the anterior end of the body is drawn out. the blepharoplasts appear sometimes one behind the other, and with a considerable interval between them.

The following is an attempt at a brief diagnosis of *Trypanoplasma gurneyorum*:—Occurs under two forms, ordinary and large; trophonucleus near middle of body, with single karyosome; kinetonucleus compact or drawn out, large; anterior flagellum of moderate length; free portion of posterior flagellum very short. Host, *Esox lucius*, Norfolk.

7. TRYPANOPLASMA KEYSSELITZI, sp. n. (Plate III. figs. 40–44; Plate IV. figs. 66–73.)

The occurrence of a trypanoplasm in the Tench has been noted by Keysselitz (Arch. Protist. vii. p. 3), but the species has not been described in detail, so far as I am aware, or named. Since it appears to me to be a well-marked species characterised by several striking peculiarities of structure, I propose to name the trypanoplasm of the Tench *Trypanoplasma keysselitzi*, in honour of Dr. Keysselitz, who was the first, I believe, to observe it. From the figure given, however, by Lühe (Mense's Handbuch, iii. fig. 5, p. 83) of the trypanoplasm of the Carp, I think it possible that *T. keysselitzi* may prove to be a synonym of *T. cyprini* Plehn.

Trypanoplasma keysselitzi was found in all the tench examined by me at Sutton Broad, always in company with *Trypanosoma tincæ*. In some of the tench examined it was very abundant. In one case I saw as many as four or five in a field (Zeiss Oc. 4 Obj. D) in a drop of fresh blood under the coverslip. In other cases the parasites were scarcer, but in no case was I unable to find them.

The trypanoplasms occur under two distinct forms: one smaller, which, as it is the most abundant, I will call the ordinary form (figs. 42, 43); the other, less common, I will refer to simply as the large form (figs. 40, 41). The two forms are easily distinguished in the living state in the fresh blood. In preparations they are seen to be distinguished not only by differences in size but also by points of structure. The posterior flagellum is continued beyond the undulating membrane as a relatively very long free flagellum in the ordinary forms, but the free portion is quite short in the large forms. There are also differences in the nuclear structure, presently to be described, between the ordinary and the large forms, and by this feature the large forms can also be separated into two types.

The cytoplasm is finely and densely granular, and in the large form opaque and deeply-staining, but it is relatively free from the coarse granules which are such a feature of *T. gurneyorum*. In iron-hæmatoxylin preparations only a few coarse granules are to be seen, sometimes none at all.

The two nuclei of this trypanoplasm are remarkable, in the first place, for being situated very close together. The trophonucleus, always placed at the edge of the undulating membrane, is never more than a short way behind the kinetonucleus, which it usually overlaps at the hinder end, and sometimes even the trophonucleus is opposite the middle of the kinetonucleus. The arrangement of the two nuclei gives this trypanoplasm a very characteristic figure and appearance.

The trophonucleus shows well-marked variations of structure. In the ordinary forms, when stained with iron-hæmatoxylin, it is a small oval clear space which contains two distinct karyosomes (figs. 70–72). These bodies may appear as small dots, looking like two granules of the cytoplasm, or they may be larger, but the number is constant, so far as my observations extend. One karyosome is often slightly larger than the other. When the trypanoplasm lies in a certain position, the karyosomes may be quite hidden under the opaque kinetonucleus, and then the trophonucleus cannot be made out at all. In the large forms, on the other hand, the trophonucleus shows either one large karyosome which almost fills the nuclear cavity (fig. 69), or one large and one or two smaller karyosomes (figs. 67, 68). There are further differences to be observed in the trophonuclei of the large forms which appear to be correlated with differences in the kinetonuclei. Usually a large trophonucleus with a very large karyosome is correlated with a relatively short kinetonucleus (fig. 69). On the other hand, a small trophonucleus may be found combined with a very long kinetonucleus (fig. 67). According to Keysselitz the large forms of the trypanoplasm are gametes, those with the large kinetonucleus being male forms, and those with smaller being female. I am not in a position to criticise or comment upon this statement.

The kinetonucleus is situated on the side of the body furthest from the undulating membrane, and is remarkable for its great length, being narrow and band-like, never rounded, oval or pear-shaped. As already stated, its length varies in different specimens. With iron-hæmatoxylin it always stains an even black and shows no structure; in one specimen which had been very much extracted (fig. 72) it appeared very narrow and linear in form, with a clear space in the cytoplasm on one side.

In front of the kinetonucleus are situated the two minute blepharoplasts from which the flagella arise. I believe them to be always two in number, but in iron-hæmatoxylin preparations they are so minute and often so close together that it is impossible to resolve them as two granules and they may appear as a single dot. There appears to be a streak or fibre connecting the kinetonucleus with the blepharoplasts, but the length of the connection, that is the distance between the two structures, is variable.

The following is an attempt at a brief diagnosis of *Trypanoplasma keysselitzi*:—Occurs under two forms, distinguished by size and by structural characters; in both forms the two nuclei very close together at the anterior end of the body; kinetonucleus very elongated, trophonucleus small; posterior free flagellum long in the smaller forms, short in the larger forms. Host, *Tinca vulgaris*, Norfolk.

8. TRYPANOPLASMA ABRAMIDIS Brumpt. (Plate III. figs. 49–54; Plate IV. fig. 77.)

The trypanoplasm of the Bream has been described by Brumpt (CR. Soc. Biol. lx. 1906, p. 164) in the following words:—

"Dans le sang de la Brême. Vu seulement à l'état frais. Le corps possède alors 30μ de long sans compter les flagelles, l'antérieur ayant environ 15μ de long, le postérieur 5 et 6μ seulement. Ce parasite évolue exclusivement chez l'*Hemiclepsis* et ne passe jamais dans la gaine de la trompe, ce qui permet de le distinguer des espèces déjà connues de la Carpe et des Loches."

The occurrence of trypanoplasms in the bream has also been noted by Keysselitz (Arch. Protist. vii. 1906, p. 3).

I found trypanoplasms fairly abundantly in all the bream examined by me at Sutton Broad. It is unnecessary to give a detailed description; my figures show their general appearance. The body is rather slender and not very granular. The anterior flagellum is fairly long, the posterior free flagellum moderately so. The trophonucleus is about on a level with the hinder end of the kinetonucleus. I did not stain any preparations with iron-hæmatoxylin, so can say nothing about the minute structure of the nuclei. I did not find any trypanoplasms of large size, as in other species.

In its characters *Trypanoplasma abramidis* appears to be more or less intermediate between *T. keysselitzi* and *T. gurneyorum*, but distinct from either.

TRYPANOPLASMA BORRELI Lav. & Mesn. (Plate III. fig. 55.)

The trypanoplasm of the Rudd (*Leuciscus erythrophthalmus*) is the type-species of the genus *Trypanoplasma*; it was described under the name *T. borreli* by Laveran & Mesnil in 1902 (Arch. Protist. i. p. 489). The Rudd and Roach are very abundant in Sutton Broad, perhaps the two commonest species of fish there, and I examined a great number of specimens of each species for blood-parasites; only in one rudd, however, was I successful in finding them. I saw one trypanoplasm in the fresh blood, and made several smears from the same fish. After very prolonged searching of the smears a single trypanoplasm was found, which both from its occurrence in the Rudd and from its resemblance to the figures of Laveran & Mesnil and Léger, I have no hesitation in identifying as *T. borreli*. From the single specimen before me, *T. borreli* appears to resemble *T. keysselitzi* in the length of its free flagella, but differs in having the trophonucleus further back (fig. 55).

EXPLANATION OF PLATES I.–V.

All figures are drawn with the camera lucida to a magnification of 2000 linear; with the exception of fig. 77, Plate IV., and figs. 94, 95, Plate V., which are drawn from the living object freehand.

PLATE I.

Figs. 1–7. *Trypanosoma granulosum* of the Eel, a series to show the gradations in size between the smallest and the largest forms. All from the same blood, and all, except fig. 7, from the same slide. Preparations dried off, then fixed with absolute alcohol and stained with Giemsa's stain.

Figs. 8–14. *Trypanosoma percæ* of the Perch, various forms. 8–11, small forms; 12, medium-sized form with long free flagellum; 13, 14, stout forms with short free flagellum; 8 & 12–14 from preparations dried off and fixed with absolute alcohol; figs. 9–11, from preparations fixed with osmic vapour followed by absolute alcohol; all stained with Giemsa's stain.

Fig. 15. Encysted form of *Trypanosoma percæ*, from the same slide as 9 and 10. Osmic vapour, absolute alcohol, Giemsa.

Fig. 16. Young form of *T. percæ*? or vermicule of a hæmogregarine? From the same slide as the last.

Figs. 17–19. Leucocytes of the Perch, showing peculiar enclosures. From a preparation dried off, fixed with absolute alcohol, and stained with Giemsa's stain.

Plate II.

Figs. 20–26. *Trypanosoma remaki* of the Pike. 20–22 & 25, small forms (var. *parva*); 23, 24, & 26, large forms (var. *magna*); 20–24, from preparations fixed with osmic vapour followed by absolute alcohol; 25, 26, from preparations dried off, fixed with absolute alcohol, all stained with Giemsa's stain.

Figs. 27–29. *Trypanosoma tincæ*, from the Tench. 27, dried, absolute alcohol, Giemsa; 28, 29, osmic vapour, absolute alcohol, Giemsa.

Figs. 30–32. *Trypanosoma abramis*, from the Bream. 30, 31, osmic vapour, absolute alcohol, Giemsa; 32, dried off, absolute alcohol, Giemsa.

Figs. 33–39. *Trypanoplasma gurneyorum*, from the Pike. 33–36, osmic vapour, absolute alcohol, Giemsa; 37–39, dried off, absolute alcohol, Giemsa.

Plate III.

Figs. 40–44. *Trypanoplasma keysselitzi*, from the Tench. 40, 41, large forms: 42–44, ordinary forms; 40–43, osmic vapour, absolute alcohol, Giemsa; 44, dried off, absolute alcohol, Giemsa.

Figs. 45–48. Large uninucleate leucocytes of the Tench, showing peculiar pink-staining enclosures; preparation dried off, fixed with absolute alcohol, stained with Giemsa's stain.

Figs. 49–54. *Trypanoplasma abramidis*, from the Bream. 49, fixed with sublimate-acetic (95 : 5); 50–52, fixed with osmic vapour followed by absolute alcohol; 53, 54, dried off, fixed with absolute alcohol; all stained with Giemsa's stain.

Fig. 55. *Trypanoplasma borreli*, from the Rudd. Osmic vapour, absolute alcohol, Giemsa.

Plate IV.

Figs. 56–65. *Trypanoplasma gurneyorum*, from the Pike. 56, large form, from a preparation stained with Giemsa's stain, all the others from preparations stained with iron-hæmatoxylin; 56, osmic vapour, absolute alcohol; 57–61 & 63, sublimate-acetic (95 : 5); 62, 64, 65, Mann's picro-corrosive with formol.

Figs. 66–73. *Trypanoplasma keysselitzi*, from the Tench; all from preparations stained with iron-hæmatoxylin. 66–69, large forms; 70–73, ordinary forms; 66, Schaudinn's fluid; 67 & 70, sublimate-acetic (95 : 5); 68, 69, & 71–73, Mann's picro-corrosive with formol.

Figs. 74–76. *Trypanosoma tincæ*, from the Tench, all from preparations stained with iron-hæmatoxylin; 74, Schaudinn's fluid direct; 75–76, Schaudinn's fluid preceded by exposure to osmic vapour.

Fig. 77. *Trypanoplasma abramidis*, from the Bream, sketched living.

Plate V.

Figs. 78–93. *Trypanosoma granulosum*, from the Eel, all from preparations stained with iron-hæmatoxylin. 78–82 & 85–93, fixed with Mann's picro-corrosive-formol mixture; 83, 84, fixed with sublimate-acetic (95 : 5).

78–80. Small forms.

81–82. Medium-sized forms; in 82 only the posterior part figured, to show the double line in the undulating membrane, the outer line representing the flagellum, the inner line representing the limit of the extension of the endoplasm into the membrane.

83. Large form, anterior half, the stain but slightly extracted, showing the cytoplasmic granules; whole length of the flagellum not drawn.

84. Large forms, anterior half, stain under-extracted, showing the myonemes; whole length of the flagellum not drawn.

85. Large form.

86–93. Trophonuclei of different specimens, all from the same preparation, and all from large forms.

Figs. 94-104. *Trypanosoma percæ*, from the Perch, all from preparations stained with iron-haematoxylin, except 94, 95, 103, 104. 97, 98, fixed with Schaudinn's fluid after previous exposure to osmic vapour; 98-102, fixed with Mann's picro-corrosive-formol; 103, osmic vapour followed by absolute alcohol, 104, dried off and fixed with absolute alcohol, both stained with Giemsa's stain.

94, 95. Sketch of *Trypanosoma percæ*, large forms, drawn living; 94, a specimen showing two dots at the hinder end (kinetonucleus and blepharoplast?); 95, a specimen showing a refringent dot, lodged in a vacuole, close behind the trophonucleus.

96. Specimen showing myonemes on both surfaces of the body, from a preparation in which the stain was but slightly extracted.

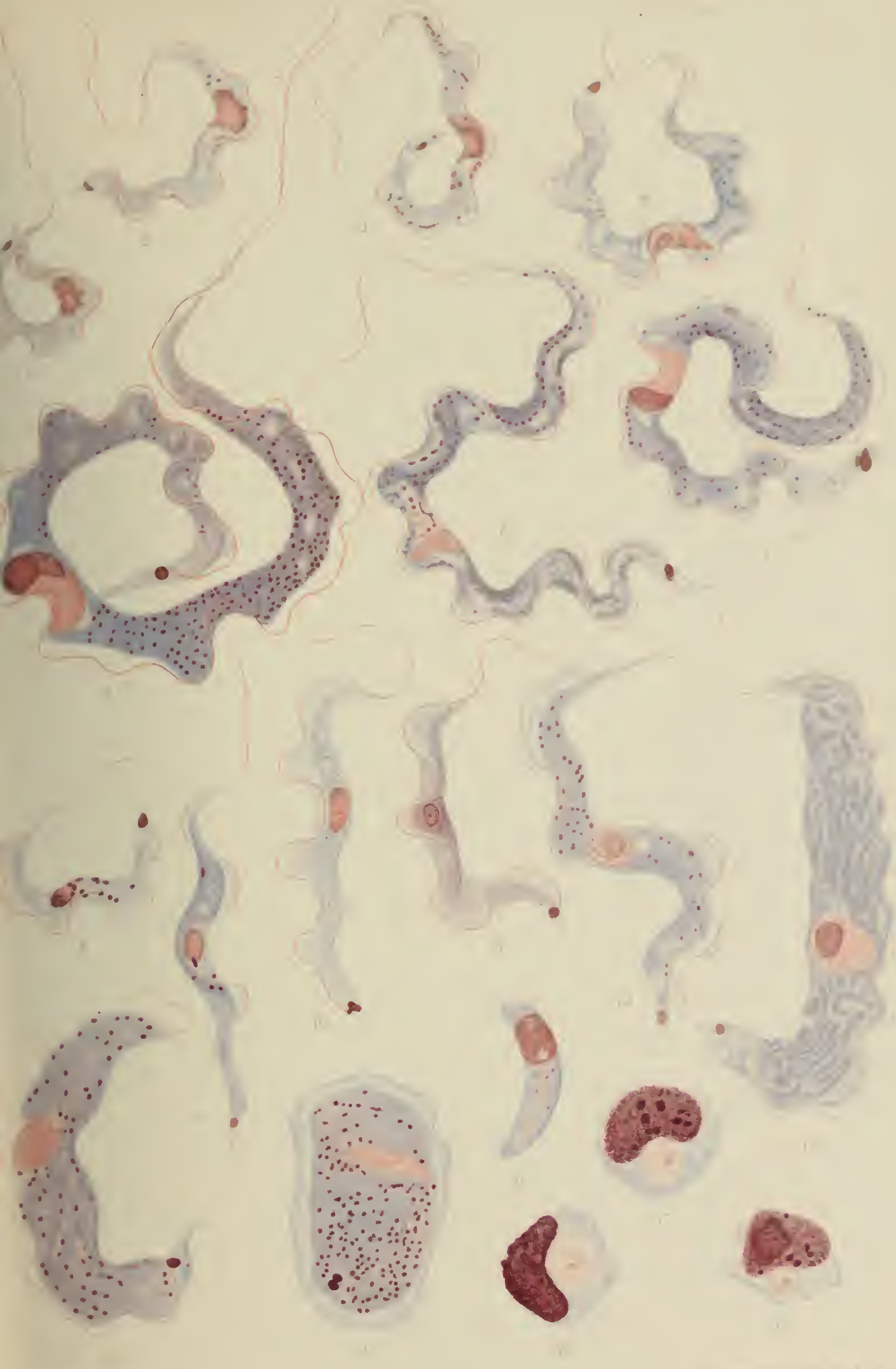
97. Middle portion of another specimen, very contracted, showing myonemes, from the same preparation as the last.

98. Specimen showing a large blepharoplast, connected with the kinetonucleus (or division of the kinetonucleus?).

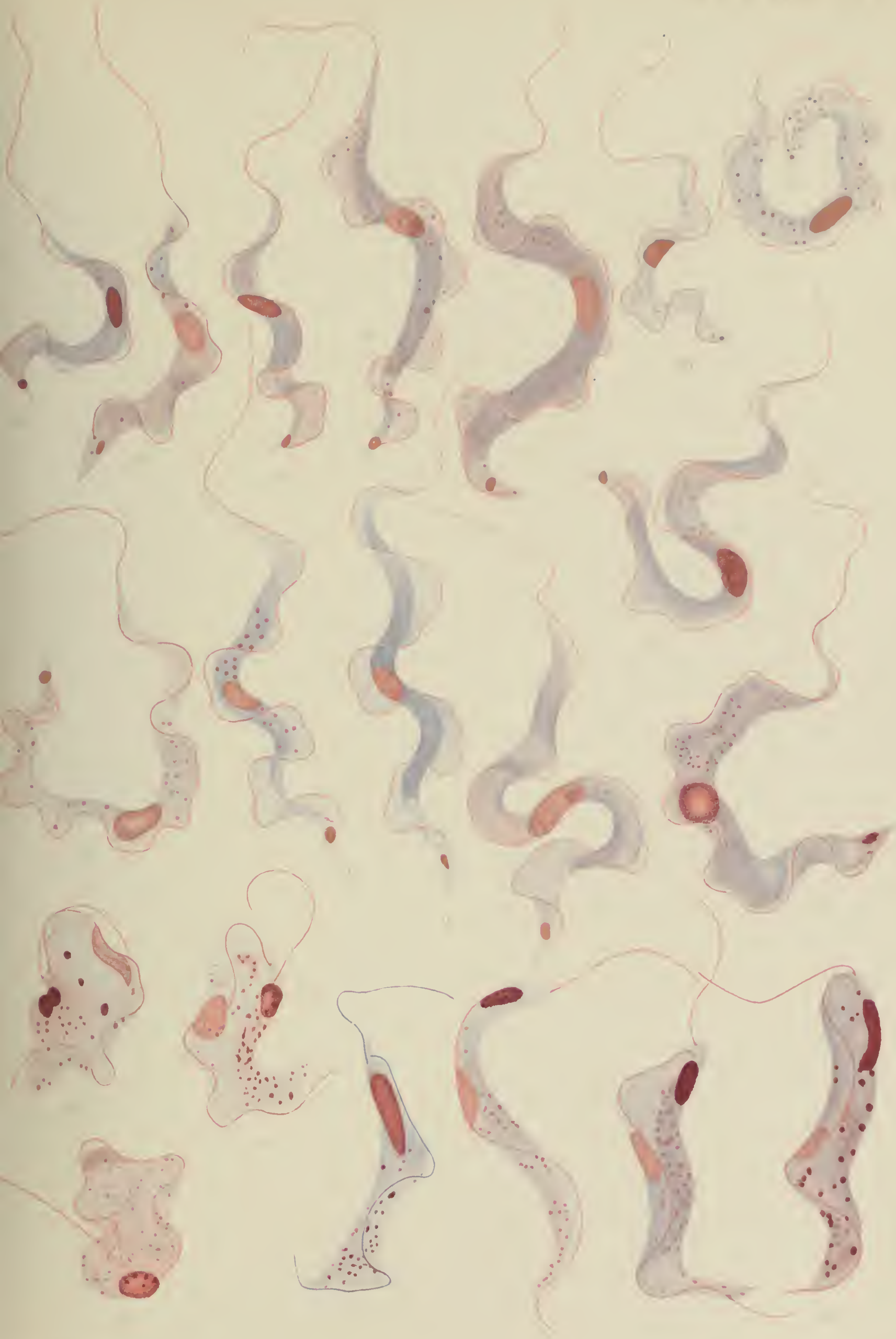
99. Another specimen from the same preparation as the last.

100-102. Trophonuclei of different specimens.

103, 104. Stout forms drawn to show the distribution of the cytoplasmic granules; in 104 many of the granules are in pairs, as if dividing or recently divided.



TRYPANOSOMES OF EEL AND PERCH



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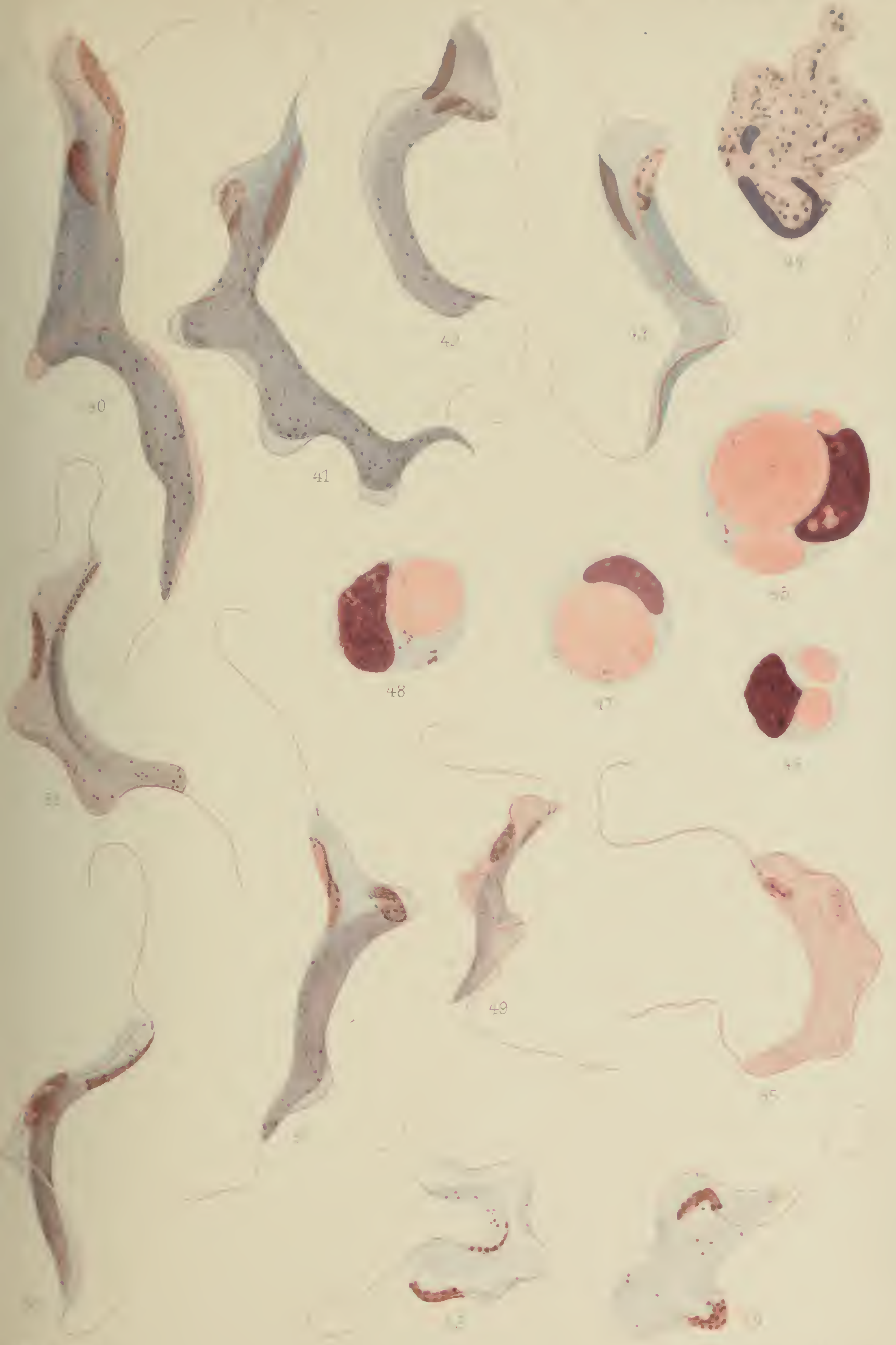
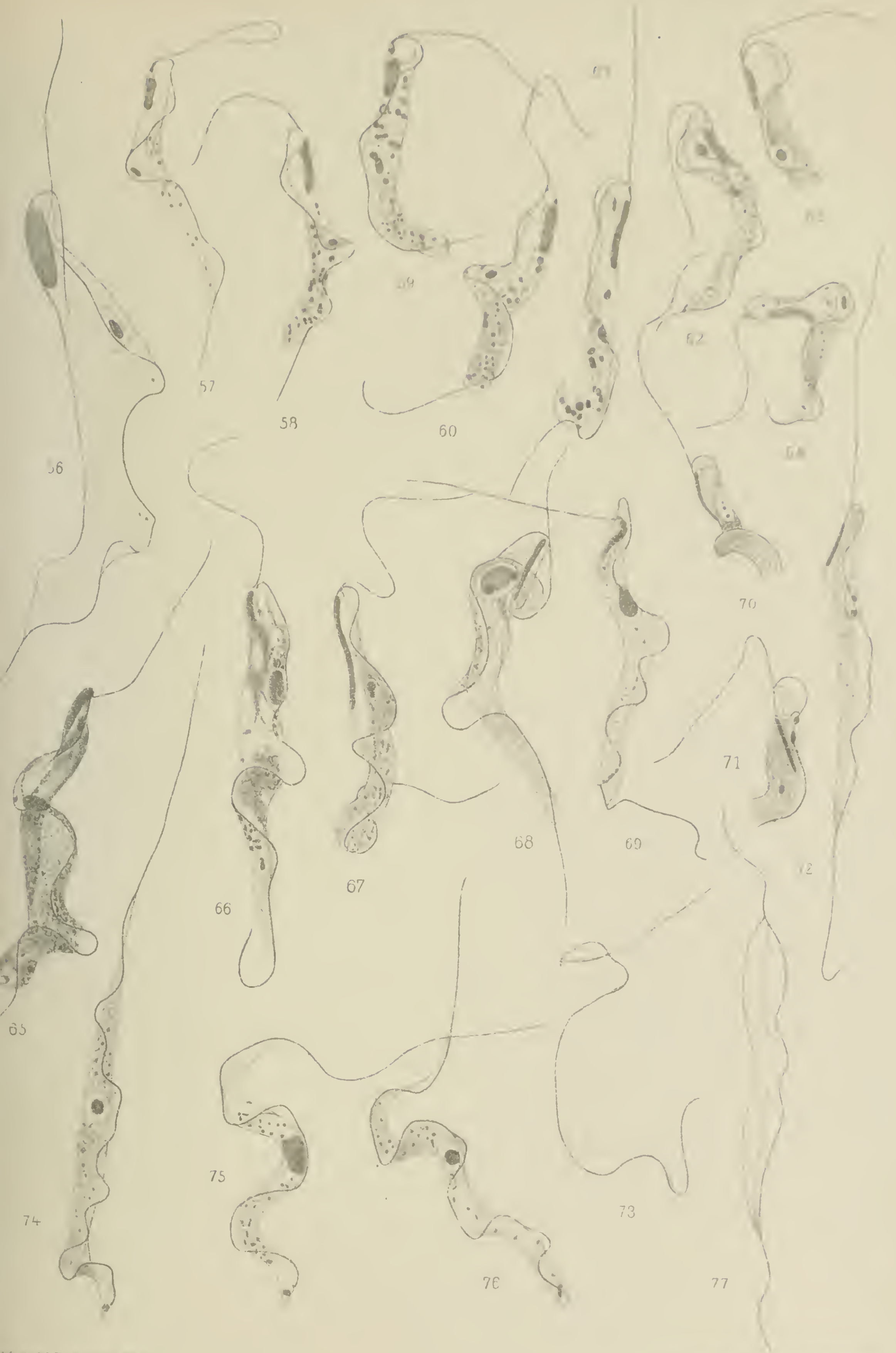


TRYPANOPLASMS OF TENCH, BREAM AND RUDD.



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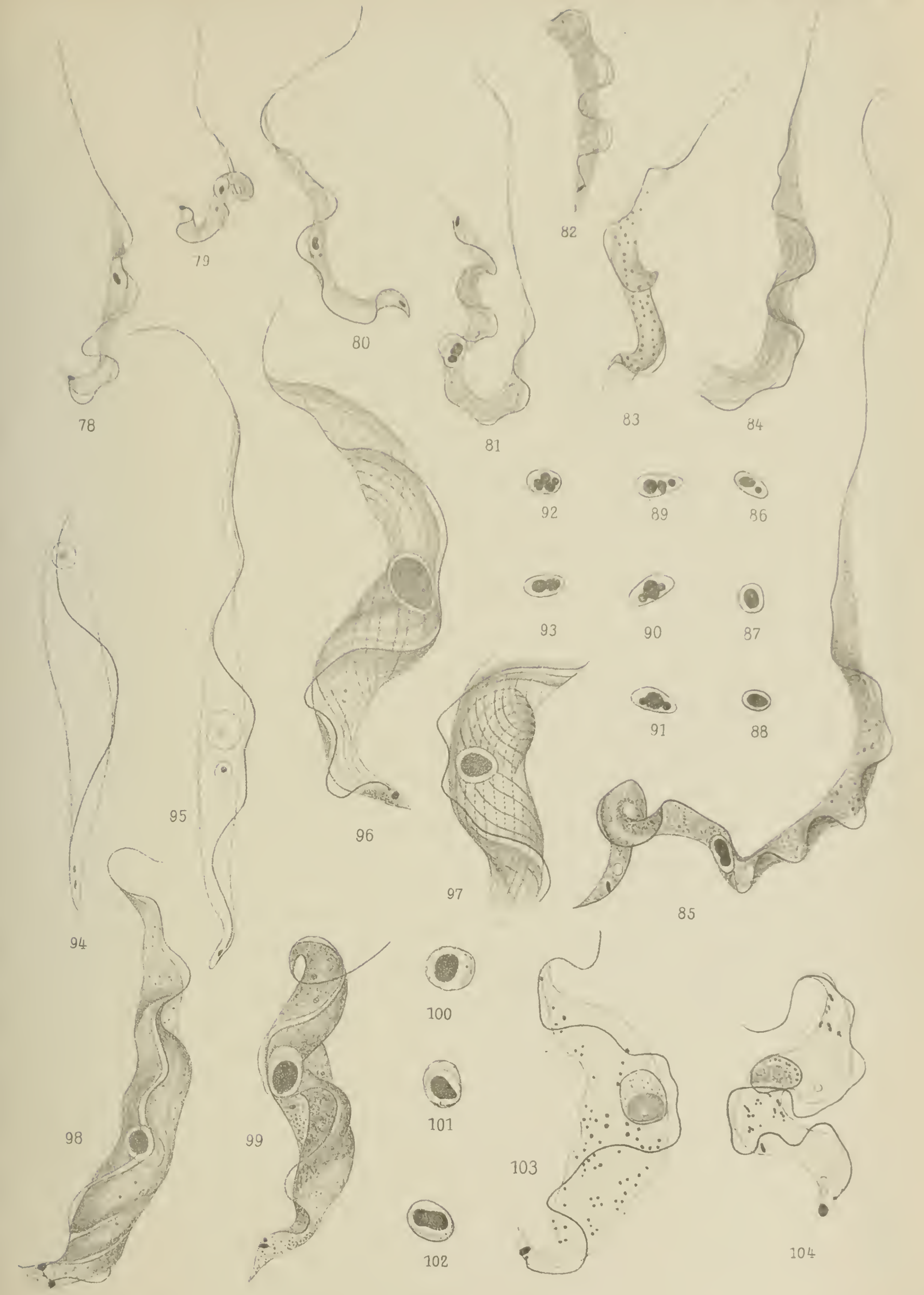
TRYPANOPLASMS OF PIKE, TENCH AND BREAM.
TRYPANOSOMES OF TENCH



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TRYPANOSOMES OF EEL AND PERCH.

The Structure of Trypanosoma lewisi in Relation to Microscopical Technique.

By
E. A. Minchin,
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With Plates 21, 22, and 23.

Introduction.

Much has been written of late years about the minute structure of trypanosomes, and also about the merits or demerits of the various methods in vogue for preparing them for examination with the microscope, that is to say, the technique of fixing, staining, and preserving these tiny creatures. Since our knowledge of the structure of trypanosomes is based almost entirely on the results of a number of complicated chemical and physical processes practised on a very delicate protoplasmic body, it is clear that a knowledge of the effects produced by these processes on the organisms are most important in interpreting the microscopic image finally obtained, in order to estimate, in any given case, how far the trypanosome may have undergone deformation or change as the result of the treatment it has gone through. In a perfect state of scientific knowledge it would, no doubt, be possible to deduce exactly such results from the known action of the reagents employed upon the protoplasmic body, but in the present condition of our knowledge it is only possible to arrive empirically at an approximate estimate of the effects of technique by comparing carefully the results yielded by it.

In order to eliminate as much as possible disturbing factors due to variability in the objects themselves, I have made use in these researches of the common trypanosome of the rat, since it can be obtained in a practically monomorphic condition and does not exhibit the remarkable polymorphism shown, for example, by the trypanosome of sleeping sickness. Moreover, after about the tenth day of infection all multiplication ceases, and though individuals may be found occasionally with two or even three trophonuclei, I am convinced that this condition has nothing to do with division, as has sometimes been supposed, but is to be regarded as an abnormality. Hence in Trypanosoma lewisi, after the multiplication-period is over, the variability in size and structure is reduced to a minimum, and the details of cytological structure are not complicated by changes due to processes of fission or multiplication.

This memoir is divided, therefore, into two parts. In the first part I shall deal with the subject from the point of view of the technique, and discuss the results obtained by different methods of fixing, staining, etc., under their respective headings. In the second part I shall discuss the structure of the trypanosome itself, taking its various parts in order. In dealing with two subjects which, though distinct, necessarily overlap to a certain extent, it is very difficult to avoid repetition, and though I shall try to steer clear of this defect in exposition as much as possible, I must crave indulgence in those parts of my subject where repetition is an alternative to omission of necessary statements.

PART I.—TECHNIQUE.

The subject of modern microscopical technique is an absolutely inexhaustible study; no one in a human life-time could claim to have said the last word upon it. As I write, all sorts of methods that I have not tried, or variations upon methods that I have tried, present themselves to my mind. It becomes necessary in a research of this kind to set a term to one's work, and to rest content at a certain period with what one has achieved, however incomplete it may appear to oneself or to others. Circumstances, into which I need not enter, oblige me to break off at the point I have reached, and to bring forward my results, such as they are, without undertaking further investigation.

The object of microscopic technique when applied to an organism such as a trypanosome is to produce a microscopic image which shall represent, so far as possible, the form and minute structure which we believe the organism to possess actually in the living state, true in all details, exact to a definite known scale of magnification, and coloured artificially so as to assist in rendering visible the different parts of the organism. A method which would have this ideal result would be a perfect method; but unfortunately no such method is known to exist, since all technique deforms or falsifies the form or structure of the organism to a greater or less extent. We are therefore confronted at the start with the difficulty, that since we can only arrive at a conception of the true and actual structure of the objects by the use of methods of technique, we are obliged to estimate the deviations from the truth, produced by technique, solely by a comparison of results which are themselves one and all defective. The only conceivable method by which the true form and structure of Trypanosoma lewisi could be recorded would be by a photograph of it in the living state; but I know of no method by which a snapshot can be taken of a minute and transparent organism, in a state of incessant and active movement, at a magnification of 2000 or 3000 diameters.

It was necessary, therefore, to find at the outset some method of killing and preserving the trypanosomes with the least possible deformation or alteration, in order to serve as a standard of comparison for other methods. It has always been my experience, and I think that of others, that in the case of Protozoa of larger size, such as Ciliata, Amœbæ, etc., the most life-like preparations can be obtained by simply exposing suddenly the living organisms, moving freely in a small drop of the medium they inhabit, to the vapour of strong osmic acid. The organisms are then examined without further treatment, except that the preparation is sealed up to avoid evaporation of the fluid medium, and in this way it is possible to obtain very perfect temporary preparations, which can be kept unaltered for at least some months. I made use, therefore, of this method for trypanosomes, but on account of the medium, blood, in which they live, special precautions were necessary in the application of it.

My first method was to take an ordinary microscope slide with a small depression hollowed out in the centre, and to cement on to this a ground-glass ring, surrounding the hollow. The upper edge of the ring was then painted with vaseline, and a drop or two of osmic acid solution (4 per cent.) placed in the hollow of the slide. Now a coverslip was taken and a drop of fresh blood placed in the middle of it and spread out with a clean glass rod, not, however, smeared out into a thin layer. It was then placed with the blood downwards on the ground-glass ring over the osmic acid, and the coverslip pressed down all round on the vaseline. All this manipulation was done with the greatest possible rapidity, in order to avoid evaporation of the blood as much as possible. Thus the blood in a hanging drop is exposed to the vapour of strong osmic acid in an air-tight chamber; it is, of course, important that the blood should not come into contact with the osmic acid solution. It is now possible to examine the trypanosomes without further treatment; but owing to the thickness of the preparation, difficulties arise in the illumination of the object, since it is not possible to focus the substage condenser properly for the use of the highest powers. I was able, however, to draw the trypanosomes in outline, at a magnification of 3000 (figs. 1, 2), but could not make out minute details. In order to do this a further manipulation was necessary.

After the coverslip with the blood had been exposed to the osmic vapour for a certain time, it was picked off the glass ring and at once placed with the blood downwards on an ordinary clean side. From the glass ring the coverslip takes with it a ring of vaseline, which when pressed down on the slide forms an air-tight cell for the enclosed blood. To ensure against the evaporation the coverslip is further luted all round the edge.[1] The blood is thus removed from the osmic chamber with no other alteration than the amount of evaporation which it suffers during the rapid passage through the air from the glass ring to the clean slide. Preparations made in this way keep without perceptible alteration for months; the trypanosomes can be examined with any power of the microscope and drawn to any scale; it is possible to make out all details of structure in them and even some which cannot be made out in other ways (figs. 3–8). The trypanosomes have undergone no changes during the treatment than such as result from the fixation with the vapour of osmic acid on the one hand, and such as may be due, on the other hand, to the slight amount of evaporation of the blood-serum which is inevitable, however rapidly the manipulation is carried out; hence these preparations represent, in my opinion, the nearest approach possible, in the present state of our technique, to the living condition, and may therefore serve as a standard for comparison with the results of other methods. I shall therefore refer briefly in the sequel to these preparations as "the standard," whenever I have to speak of them.

In making the standard preparations I introduced one additional complication into some of them, namely, that on the clean slide a small drop of acidulated methyl-green solution was placed ($\frac{1}{2}$ per cent. methyl-green in 1 per cent. acetic acid), and the blood, after exposure to the osmic vapour, was placed on the stain. In the course of a few hours the stain mixes with the blood and tinges the nuclei of the trypanosomes. Comparison of trypanosomes, treated with the acidulated methyl-green (figs. 5–8), with those in which no stain was used (figs. 3, 4), revealed no perceptible difference in size, form or general structure,[1] so that no ill-effects result from the use of the stain, while the microscopic image is rendered much sharper and clearer.

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[1] I employ for this Czokor's mixture of beeswax and Venetian turpentine, heated together until the mass is hard and smooth (not sticky) when cool, and applied to the edge of the coverslip with a piece of heated wire.

My method of carrying out investigations upon the effects of technique was the following: Preparations of Trypanosoma lewisi were fixed and stained in various suitable ways, and the results were all drawn with the camera lucida at a magnification estimated at 3000 diameters, and are reproduced in this memoir at the same scale. It is possible that the figure 3000 is not perfectly accurate, but if not, it makes no difference to the result, since the object was to compare the effects of the methods employed, and for purposes of comparison the exact magnification is immaterial, provided it be uniform throughout. All the drawings given here were executed using always the same microscope and length of tube, the same lenses, camera lucida, drawing board and illumination. Some of them were done by me, but most of them by my assistant, Miss Rhodes, to whom my best thanks are due for her skilful help. Zeiss's apochromatic objective 2 mm., 1·40 aperture, was used, combined with compensating oculars. The source of illumination was the flame of an oil lamp placed edgeways, and concentrated by a Zeiss collecting lens, through a monochromatic screen on to the mirror of the microscope, and thence reflected through a centring achromatic condenser of Zeiss. This illumination, if properly arranged and focussed, gives very perfect definition—a very important point in studying these preparations; I have frequently found that structures had been overlooked which became visible with improved illumination. I frequently compared the results obtained in this way with those given by a monochromatic illuminating apparatus set up with a prism in such a way that any part of the spectrum could be used, in order to obtain confirmation of the structures as drawn with the illumination described.

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[1] A slight vacuolation, which is probably an artefact, makes its appearance near the kinetonucleus after treatment with methyl-green (figs. 5–8).

Before proceeding to describe in detail the results of the fixatives and stains used by me, I will say a few words about the methods of applying them. I began with the ordinary method of making smears on slides; this is quite suitable for smears that are to be dried off or fixed with osmic acid vapour, for which the procedure adopted was the following: a glass tube is taken, of suitable size and calibre, and provided with a tightly fitting cork or stopper; at the bottom of the vessel are put twenty drops of 4 per cent. osmic acid solution with one drop of glacial acetic; when the smear has been made, the slide bearing it is placed with as little delay as possible into the tube containing the osmic acid and corked up. It is advisable to take precautions against any part of the blood-smear coming into contact with the osmic acid solution, if one wishes to be economical in the use of this most expensive re-agent. An exposure of thirty to forty-five seconds to the osmic vapour is sufficient; the fixation is practically instantaneous. Slides fixed with osmic vapour in this way may be (1) dried off, then fixed with absolute alcohol or methyl alcohol in the ordinary way; or (2) placed at once without drying into absolute alcohol, stained as desired and then dried off; or (3) fixed, stained, and mounted in Canada-balsam without ever having been dried.

I soon, however, abandoned the use of slides for smears for various reasons. In the first place it is a clumsy method for rapid manipulation, and however anxious one may be to shorten the exposure to the free air, it is very difficult to avoid a certain amount of drying taking place in such large smears, either at the edge or in thin places. In the second place, slide smears do not give good results when the fresh wet smear has to be fixed by sudden immersion in a liquid, which it almost necessarily enters with a dive, so to speak, with one end foremost; in such cases it is common to find all the blood-corpuscles drawn out in an extraordinary manner, and all the trypanosomes stretched out straight in one direction, indicating the line of the dive into the fixative. In the third place I may point out that since the best immersion objectives are corrected for coverslips, naked smears without coverslips do not give optical results so good as those covered, and that in covered smears it is optically preferable for the smear to be on the coverslip rather than on the slide.

The majority of the preparations described here were done by Schaudinn's method of making smears on a coverslip and then dropping the coverslip flat down plump into the fixative. I hold a coverslip in the fingers of my left hand, a glass rod in my right; in front of me is the liquid to be used for fixation. An assistant draws the drop of blood from a rat's tail, and either places it on the coverslip I am holding, or I take it up on the end of the glass rod from the tail; in either case I smear the blood out with the glass rod and drop the coverslip, with the smear downwards, into the fixative. The whole process is done with one turn of the right wrist and one of the left, far more rapidly than a slide can be manipulated and with much less chance of drying or time for evaporation to take place. Coverslip smears made in this way are always stained and mounted in Canada-balsam without being allowed to dry during any part of the process. They are most conveniently handled in solid watch-glasses (that is to say, a square slab of plate glass, two and a half inches square and a quarter of an inch in thickness, hollowed out on one side into a cavity the size of a watch-glass); the coverslip can be placed in this with only sufficient liquid to keep it wet on the underside (the side of the smear), and can be examined under the microscope with moderate magnification at any time, and the glasses can be stacked one on the top of the other to prevent the contents drying up.

The use of coverslip smears necessitates a modification in the mode of applying the osmic acid vapour for fixation of films. I take a square block of hard paraffin of suitable size, and a glass ring ground flat on the two sides. The glass ring is gently heated and so cemented to the middle of one surface of the block of paraffin, which is then hollowed out into a small cell inside the glass ring. The osmic acid is placed in the cell in the paraffin, and the coverslip, with the blood-smear downwards, is placed on the glass ring, to which it sticks by means of the wet blood itself, and makes a practically air-tight chamber. A certain amount of the smear has to be sacrificed, of course, by this method, but there is always enough for all practical purposes preserved within the ring. After sufficient exposure the coverslip is lifted carefully off the ring and dropped at once into absolute alcohol or other fixative. In this method the only precaution necessary is the protection of the operator's eyes and air-passages from the vapour of the osmic acid. It is advisable either to wear protective spectacles or to have the osmic cell covered with a glass plate, which an assistant removes the instant it is desired to place a coverslip on it.

Salvin-Moore and Breinl (1907) recommend making smears on slides previously covered with a thin layer of glycerine and albumen, but with what object I do not understand, as the blood-films always stick on perfectly well to the coverslips and slides when plunged suddenly into fixatives, and neither corpuscles nor trypanosomes come off. Even when working with fish-blood, in which the parasites are exceedingly scanty, I have always found them in my never-dried smears just as abundantly as in those done by other methods. In our technique the frail bodies of these unfortunate creatures are subjected to so many processes of violent treatment that to avoid deformation it is better to reduce and eliminate these processes as much as possible rather than to increase them. The addition of glycerine and albumen to the fresh blood containing living trypanosomes can but be an additional source of error, and is therefore, in my opinion, best avoided.

I made several attempts to obtain preparations of trypanosomes by the method recommended by Schaudinn (1902, p. 190) for malarial parasites, that is to say by dropping fresh-drawn blood into fixatives, such as Flemming's fluid or Schaudinn's fluid, in a centrifuge tube, and then carrying out all the subsequent processes of washing, staining, etc., by means of the centrifuge. After much searching I found some trypanosomes in preparations made in this way, but they were so shrunk and deformed as to be scarcely recognisable.

When preparations that have been mounted in Canada-balsam without being dried at any time are compared with those that have been dried off at some stage of the process, either before fixation, or after fixation with osmic vapour, or after staining, it is found that the trypanosomes in the never-dried preparations are constantly slightly smaller than in the dried-off preparations. Comparison with the standard shows that the dried-off trypanosomes have lost very little in bulk, while the never-dried trypanosomes have lost considerably. I obtained invariably the same results with the trypanosomes and trypanoplasms of fishes; the never-dried specimens mounted in Canada-balsam were always a size smaller than those in the dried-off films. This result proves, in my opinion, that the changes of medium which are incidental to the processes of dehydration with alcohol, clearing with xylol or other media, and mounting in Canada-balsam, have the effect of diminishing the size of the body. So long as it is kept in a semi-fluid plastic condition the body is susceptible to changes of medium, and can be shrunken or swollen by them. If, however, all fluid be once removed from the body by evaporation, it appears to obtain a rigidity of texture which resists further deformation, although it may be altered in form to a greater or less extent by the process of drying itself.

After these general remarks I proceed to describe in detail the effects produced on Trypanosoma lewisi by various fixing and staining methods, using as the standard of comparison the preparations fixed simply with osmic acid vapour and examined in the fluid blood, as described above.

(1) Fixatives.

The fixatives I have used may be grouped conveniently under the following heads: (1) Osmic acid vapour, followed simply by alcohol; (2) mixtures containing osmic acid, (3) mixtures in which corrosive sublimate is the principal ingredient; (2) and (3) were either used directly on the wet film, or after previous exposure to osmic acid vapour.

(1) Osmic acid.—I have employed osmic acid as a fixative of the films followed by various other fixatives, such as absolute alcohol, Flemming's fluid, sublimate-acetic, etc. I will only deal in this section with its action when followed by alcohol, and discuss its action when combined with other fixatives in the sections dealing with those fixatives. Of all the methods I have tried, fixation with osmic vapour followed by fixation with alcohol (figs. 60–71) gives the best result as regards size, form, and general characters of the trypanosomes, so far as can be judged from a comparison with the standard (figs. 1–8). The osmic acid was used in the form of vapour given off from a 4 per cent. solution, combined with acetic acid in the following proportions: a 4 per cent. solution osmic acid, 20 drops, glacial acetic acid, 1 drop.

The smears, after exposure to the osmic vapour, were in some cases allowed to dry off, either before further fixation or after staining; but as a rule they were transferred at once to absolute alcohol, and were stained and mounted in Canada-balsam without being allowed to dry at all. I will distinguish these two methods of treatment briefly as the dried-off osmic method and the never-dried osmic method respectively. The dried-off method is most convenient for smears made on slides; for the never-dried methods it is better to make the smears on coverslips. The dried-off osmic smears stain very well by the ordinary Romanowsky methods (Giemsa's stain, azure-erythrosin, etc.), but I have never got good results with the iron-hæmatoxylin stain after drying off. The never-dried smears stain well with Romanowsky stain, and fairly well, but rather faintly, with iron-hæmatoxylin. The details of the flagellum and blepharoplast are difficult to make out after osmic-absolute fixation and iron-hæmatoxylin staining; for these points fixation in sublimate mixtures is far superior.

After the dried-off osmic method the trypanosomes do not show what I will term briefly a "periplast-line," that is to say, a clear border under the periplast, causing the periplast to stand out distinctly from the body (see below). By the never-dried method, however, a periplast-line appears very distinctly in the iron-hæmatoxylin preparations (Pl. 21, figs. 10, 11). Its appearance in the preparations stained with the Romanowsky stain depends largely on the degree to which the stain has been extracted by the acetone during the process of mounting in Canada-balsam, and the result varies greatly in one and the same slide (Pl. 22, figs. 64–69); a matter which I shall discuss more fully in the section dealing with this stain and its effects. One of the most constant results of the process of drying off is seen in the relations of flagellum, blepharoplast and kinetonucleus. In never-dried preparations the flagellum starts from close to the kinetonucleus, with which the blepharoplast is nearly in contact. In dried-off preparations, on the other hand, there is a distinct interval between blepharoplast and kinetonucleus, almost equal to the width of the latter in many cases (Pl. 22, figs. 60, 61). The same result is seen in preparations in which osmic fixation is followed by Flemming's fluid or other reagents, and dried off after staining with Romanowsky's stain, and also in preparations dried before fixation. It follows, therefore, that the effect of drying is to draw apart kinetonucleus and blepharoplast, probably as the result of a longitudinal contraction in the flagellum or in its basal portion.

(2) Osmic acid mixtures.—The two famous combinations, Flemming's fluid and Hermann's fluid, were used, the former both direct on the wet film and after previous exposure to osmic vapour, the latter only directly on the film. The Flemming's fluid used was the so-called strong mixture; the Hermann's fluid was made up in the same manner, and differed only in the substitution of platinum chloride (so-called), 1 per cent., for the chromic acid, 1 per cent. A variety of staining methods was tried on these films; I will describe the results obtained by the various stains in the sections dealing specially with them below. The best results were obtained with iron-hæmatoxylin, and the following account of the action of these two fixatives refers more particularly to

preparations stained by this method, unless the contrary is stated.

When used directly on the wet films both fixatives gave very similar results. The trypanosomes are under-sized and deformed, appearing too short in proportion to their breadth, as if contracted in the longitudinal direction (figs. 36–38, 40–44); their attitudes in the preparations look strained and unnatural, as if their end had not been a peaceful one. The amount of the deformation is distinctly greater after Hermann's fluid than after Flemming's, and with the latter re-agent it varies in different preparations. I am inclined to think that the amount of deformation is related in an inverse manner to the degree to which the films have lost moisture previous to fixation. However carefully and rapidly the preparation of the film be carried out, it is clear that a thin film of blood must lose a certain amount of moisture before it reaches the fixative. My impression is that the more the film dries before coming into the fixative, the less the trypanosomes undergo shrinkage and distortion of the body-form.

As regards minuter details, the following points are to be noted: The body of the trypanosome does not show a periplast-line; the nucleus has an appearance most characteristic of the effects of these fixatives; it is surrounded by a clear space, often very distinct, but not sharply limited (figs. 36–38, 43, 44), which I regard as the result of shrinkage. In the nucleus the karyosome is very distinct, and frequently also other coarse chromatic granules are to be seen. A similar condition of the nucleus could be made out also in trypanosomes stained with all the other staining methods that were used, and must, therefore, be attributed to the action of the fixative.

The kinetonucleus and blepharoplast appear normal after Flemming's and Hermann's fluids, but the flagellum often appears extremely crinkled, with sharp bends and angles, very different from the normal smooth undulating curves; its appearance is strongly suggestive of longitudinal shrinkage.

In extreme cases (Pl. 21, fig. 38) the appearance of the flagellum recalls that of a wire that has been untwisted from the neck of a soda-water bottle.

In films fixed with osmic acid vapour before being put into Flemming's fluid, I find but little difference from those fixed with absolute alcohol after the osmic treatment, except that the trypanosomes are rather broader in proportion to their length (figs. 80, 81). The nucleus stained with Giemsa's stain is a large red patch, as large as, or even larger than, the whole clear space seen after direct fixation with Flemming's and Hermann's fluids. When stained with iron-hæmatoxylin, after Flemming's fluid preceded by exposure to osmic acid vapour (fig. 39), the nucleus of the trypanosome does not show the clear space round it described above, but appears as a broad oval or nearly circular in outline. The body shows no periplast-line and the flagellum has the normal appearance. Previous exposure to osmic acid vapour would appear, therefore, to obviate some of the defective results obtained by the direct action of Flemming's fluid.

(3) Sublimate mixtures.—Three different sublimate mixtures were used for fixation: (1) Sublimate and acetic acid, (2) Schaudinn's fluid, (3) Mann's picro-corrosive mixture. All three were used directly on the wet film. The first two were used also after previous exposure of the wet film to osmic vapour. In all cases the fixing solution was allowed to act for about half an hour on the film.

(a) Sublimate acetic.—This was used in the proportion of 95 parts (by volume) of corrosive sublimate solution, saturated in distilled water, and 5 parts of glacial acetic acid. A mixture was also tried of 99 parts corrosive sublimate solution to 1 part of glacial acetic, but was found to produce very marked shrinkage in the trypanosomes (Pl. 21, figs. 12, 13) and was not used again.

The fixative was used either directly on the films (figs. 19–22, 82–84, 88–91), or after previous exposure to osmic vapour (figs. 14–18, 75), in both cases without drying before putting in the sublimate solution. The fixed films were stained in various ways, principally with the Romanowsky stain, with iron-hæmatoxylin, and with Twort's stain. Some of the films stained by the Romanowsky method were dried off (Pl. 22, figs. 70, 73), but as a rule they were kept wet and passed through acetone and xylol into balsam. All films stained with iron-hæmatoxylin or with Twort's stain were kept wet throughout and mounted in balsam.

After previous fixation with osmic vapour the trypanosomes generally show a distinct periplast line (figs. 17, 18, 75), not to be seen when the sublimate mixture is used directly. There is little other difference to be noticed between osmic-fixed films and those in which osmic has not been used. With either method iron-hæmatoxylin gives good results; Twort's stain, however, does not work well after osmic fixation, but stains very well after fixation direct in the sublimate mixture (figs. 82–84).

(b) Schaudinn's fluid (two volumes of saturated solution of corrosive sublimate in water, one volume of absolute alcohol, with the addition of a few drops of glacial acetic).—This fluid was also used with (figs. 23, 24) or without (figs. 25–28, 76, 85) previous exposure of the film to osmic vapour. The films were never dried and were mounted after staining in Canada-balsam. The results were on the whole similar to those observed with sublimate acetic. A periplast-line can generally be observed, and appears to depend on the degree of extraction of the stain, as is well seen after iron-hæmatoxylin (figs. 23–38). Twort's stain gives very good results if the fixing fluid be used without previous osmic vapour-fixation (fig. 85).

(c) Mann's picro-corrosive.—Made up as follows: 2·5 grm. of corrosive sublimate dissolved in 100 c.c. of boiling distilled water; when dissolved, 1 grm. of picric acid added; the mixture allowed to cool, and either used as made up, or with addition of 15–20 c.c. of formol, mixed in immediately before use. Wet films were fixed in the mixture and stained in various ways without drying at any time. I could perceive very little difference resulting from the presence or absence of formol, but got the impression that the fixation was rather better, and the stain sharper, after use of the mixture containing formol.

Mann's fluid gave me very uniform results. The trypanosomes show no periplast-line and appear slender. The blepharoplast and flagellum show up with remarkable clearness, especially after iron-hæmatoxylin, and this appears to me the method of choice for the study of these structures.

With all the sublimate-containing mixtures that have been tried, a marked diminution of size is apparent in the never-dried preparations as compared with the type (compare especially figs. 1–8 with figs. 16, 35, and 79). The shrinkage is on the whole greatest after the picro-corrosive mixture; the disappearance of the periplast-line after fixation with picro-corrosive indicates, perhaps, a certain amount of collapse in the cytoplasmic body.

As already pointed out above, the shrinkage is scarcely perceptible if the trypanosomes have been allowed to dry off after exposure to the osmic vapour before fixation in the sublimate mixture. Figs. 70 and 73 show this plainly when compared with figs. 14, 15, 16, etc.

The facts indicate further that the shrinkage is due not only to the action of the fixative but also to the subsequent processes which the trypanosomes pass through, namely, first the staining process, secondly the dehydration and clearing preparatory to mounting in Canada-balsam. It can be observed, for example, that the Romanowsky stain, when used on trypanosomes which have not been dried, seems to shrink them more than do other stains, such as iron-hæmatoxylin or Twort's stain; compare, for instance, figs. 19, 20, 82–84 with figs. 21, 22, from a film which, after having been fixed and stained in Giemsa's stain without drying, was dried off after the stain; the trypanosomes are shrunk to a ludicrous extent in the latter case.

Although the trypanosomes are distinctly diminished in size and general proportions after sublimate fixation (when never dried), the shrinkage appears to take place evenly, and the trypanosomes are scarcely if at all deformed. The body maintains an even, slender form, differing only from the type in size. The minute details of the nuclei, blepharoplast, and flagellum are very well seen after sublimate mixtures, and especially after picro-corrosive, better, indeed, than with any other fixative in my opinion.

(2) Staining Methods.

I have made trial of a number of staining methods on films preserved in various ways, and have obtained results which may be considered from two points of view. Some staining methods give good results generally, and yield what I may term "show" preparations — that is to say, preparations which one would not be ashamed to demonstrate to students or to strangers. Other stains give results which are quite useless considered as preparations for purposes of demonstration, but which may be quite interesting as micro-chemical reactions. I will discuss in detail the three staining methods that have given me the best results generally, namely the Romanowsky stain in its various modifications, Heidenhain's iron-hæmatoxylin method, and Twort's combination of neutral red and licht-grün; after which I will deal briefly with other stains I have tried.

(1) Romanowsky stain.[1]—In applying this method I have always used Giemsa's modification; I obtain the stain made up in the fluid condition from Grübler, and for actual use I mix the stain with distilled water in the proportion of one drop of the stain to 1 c.c. of water. The preparations are left in from three to eighteen hours. Osmic-fixed preparations are apt to stain very darkly, and require a much shorter time than those simply fixed with alcohol. The preparations when taken out of the stain are washed with distilled water, treated for a few seconds with Unna's tannin-orange (obtained from

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[1] I use the term "Romanowsky stain" as a general term for the combination of stains, of which the methods of Leishman, Giemsa, etc., are special modifications, in application or substance.

Grübler), and then washed in a current of tap-water for a minute or two. After this the preparation is washed with distilled water, and either dried off, or passed rapidly through three changes of acetone into xylol, and mounted in Canada-balsam.

Some preparations were also stained for me by Dr. J. D. Thomson with a mixture of azure and erythrosin in the following manner: A mixture was made of (1) azure I, 1 per cent., in equal parts of glycerine and methyl alcohol, 1 volume; (2) erythrosin, 0·1 per cent., in 0·25 per cent. formol, 2 volumes; (3) distilled water, 8 volumes. The two staining fluids, kept ready in solution, are mixed with the water immediately before use, and the mixture is allowed to act for from half an hour to one hour; it works better if kept at about blood-temperature. After staining, the preparations are either washed with distilled water only, or also with tannin-orange followed by tap-water, as described above, and then either dried off or mounted without drying in Canada-balsam, passing through acetone and xylol.

The azure-erythrosin mixture stains the trypanosomes in colours slightly different from that resulting from Giemsa's stain. The body is more purple in tint, and the red colour of the nucleus and flagellum appears rather deeper after azure-erythrosin. The general effects of the two methods are the same, and do not require to be discussed separately.

The Romanowsky stain is undoubtedly the best method for studying the general characters of the trypanosomes, especially if combined with osmic fixation, which, as I have shown, preserves very well the form and structure of the body and results in the minimum of shrinkage and deformation. I have always got the best results after osmic vapour followed by absolute alcohol; when the osmic is followed by other histological reagents, such as Flemming's fluid or sublimate-acetic, the results are inferior, but still usually quite good. On the other hand I have never got good preparations with the Romanowsky stain after wet fixation in hardening fluids without previous exposure to osmic vapour; the trypanosomes become shrunk and deformed, often very greatly, and the stain is blotchy and irregular. That has been my experience in all cases with smears fixed in Flemming's fluid, sublimate-acetic, Schaudinn's fluid, Mann's fluid, etc., stained with Giemsa's stain, and mounted without drying in Canada-balsam or dried off. Preparations made in this manner are usually quite useless, while the companion slides, stained with iron-hæmatoxylin and mounted in the ordinary way, are very good.

The Romanowsky stain has the further advantage of being easy to apply and comparatively rapid in action. It is therefore the method of choice for workers to whom time, or laboratory equipment, are material considerations—that is to say, to those dealing in a limited time with large quantities of material, or working under disadvantages of installation, as in the tropics. Nevertheless I shall try to show that, as Schaudinn[1] has already stated clearly, the Romanowsky stain has some glaring defects in its action on minute structural details, and that some false conclusions and interpretations have been based upon its results. It is very important that its effects should be studied carefully and compared with those yielded by other methods, for only in this manner is it possible to eliminate errors and to discount false appearances produced by it, and to obtain, so to speak, a constant formula for estimating and interpreting the results of its use.

The peculiarities of the Romanowsky method of staining, as regards its effects on minute structures, are at once apparent when trypanosomes stained by this method are compared with others, fixed in precisely the same manner, but stained with iron-hæmatoxylin. The differences are very great, and often very puzzling; compare, for example, fig. 73, and figs. 14, 15; figs. 80, 81, and fig. 39; figs. 62, 63, and figs. 10, 11. To begin with the nuclear structures, the kinetonucleus appears much larger after the Romanowsky stain than it does after iron-hæmatoxylin, perhaps four times as large; the same two types of form are to be made out in the kinetonucleus, but they have become magnified, as it were, in the former case. The trophonucleus is not only much larger after staining by the Romanowsky method, but its structure appears quite different from that seen after iron-hæmatoxylin and other methods. In the place of a simple oval vesicle-like structure containing a deeply staining karyosome, we find after the Romanowsky stain an opaque mass of deeply stained coarse grains of reddish colour, in which a karyosome often cannot be made out with certainty. Sometimes the stained grains are closely packed and scarcely distinguishable individually; sometimes, and especially in preparations dried before fixation, the grains appear separate and distinct. Very frequently the nucleus shows a clearer spot at or near the centre (figs. 70, 80, 81). The flagellum appears thick and coarse and the blepharoplast is usually fairly large and distinct after the Romanowsky stain.

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[1] "Nur ist sie (die Romanowsky'sche Färbung) mit Vorsicht zu benutzen, weil sie oft überfärbt und Strukturen vortäuscht, die garnicht vorhanden sind. Ohne Kontrolle durch Hämatoxylin darf man keine Schlüsse über Kernstrukturen bloss nach Romanowsky-Präparaten ziehen." Schaudinn (1902), p. 191.

What is the explanation of these differences? The large size of the kinetonucleus and trophonucleus and the thickness of the flagellum after the Romanowsky stain are shown by the standard (figs. 1–8) to be a case of actual enlargement or thickening, not to be explained by supposing that after other methods, such as the iron-hæmatoxylin stain, these structures are shrunk. Such an enlargement can only be explained by a tendency of the red stain or stains, which are active in the Romanowsky combination, to deposit not only in, but also around, the structures that are coloured by them. Comparison of the kinetonucleus, in preparations stained by the Romanowsky method and by other methods, is alone sufficient to prove conclusively that the enlargement is due to this cause, and gives a clue to the interpretation of the very remarkable and perplexing differences seen in the structure and appearance of the trophonuclei in the two cases.

In order to discuss the effects of the Romanowsky stain on the trophonucleus, it is necessary that I should anticipate here the conclusions to which I come further on in this memoir with regard to the minute structure of this body in Trypanosoma lewisi. The trophonucleus is a rounded oval body with a distinct limiting envelope, which is not to be regarded as a true nuclear membrane in the sense in which this term is used for metazoan nuclei, but, in all probability, as a condensation at the periphery of the chromatin-substance itself. Inside this envelope is a space, filled doubtless in the living animal by a nuclear sap, in which are contained other chromatin-bodies; first of all the conspicuous karyosome, sometimes double or further subdivided, which in its staining reactions resembles the kinetonucleus, and appears of dense texture; secondly, smaller granules of chromatin, some of which may be fairly large and plainly visible, but which for the most part appear to be minute and not capable of being resolved by the microscope as distinct structures, but are scattered in a state of fine division throughout the nuclear sap, giving to the trophonucleus the even dark grey tint which it shows in an iron-hæmatoxylin preparation at a certain degree of extraction, or the pale red tint which it exhibits after Twort's stain.

Applying the conclusion stated above, namely, that the red stain of the Romanowsky mixture deposits round the structures it stains, to the conception of the structure of the trophonucleus that I have put forward, we can explain the results of the stain as follows. The deposition of the stain round the nuclear membrane accounts for the apparent increase of size of the nucleus as a whole. At the same time the stain is deposited round the minute granules of chromatin within the nucleus, enlarging them to relatively coarse grains. The karyosome doubtless shares also in the enlargement, but is generally obscured by the closely packed and secondarily enlarged chromatin grains, and is consequently very difficult to make out clearly.

In support of the above conclusion I refer to the series of figures drawn from three preparations (*a*, *b*, *c*) which were made at the same time from the same rat, and which were all fixed in the same manner, namely by osmic vapour followed by absolute alcohol, without drying at any time; *a* (figs. 64–69) and *b* (figs. 62, 63) were stained with Giemsa's stain, and passed through acetone and xylol into Canada-balsam; *c* (figs. 10, 11) was stained with iron-hæmatoxylin and mounted in the ordinary way.

In preparation *b* (figs. 62, 63) the passage through the acetone was evidently effected without any extraction of the stain taking place; the trypanosomes are perfectly similar in general appearance to those stained with Giemsa and dried off (figs. 60, 61). In preparation *a* (figs. 64–69), on the other hand, a considerable amount of extraction has taken place, which, however, is different in degree in different parts of the preparation; in one place a clump of trypanosomes may be found, all showing a deeply stained, opaque red trophonucleus; another patch of trypanosomes will show the stain greatly extracted. Hence in this preparation I have been able to find every stage of the extraction of the stain, and have put together the series shown in my figures.

The series begins with trypanosomes, in which the trophonucleus differs only from those in preparation *b* by its smaller size (fig. 64), showing that the process of extraction begins by removing the stain deposited outside the nuclear membrane; at the same time the kinetonucleus is considerably diminished in size, the periplast-line begins to stand out, and the flagellum is thinner. In the next stage (fig. 65), the trophonucleus is pale with a few scattered coarse granulations, amongst which it is still difficult to identify the karyosome. In the next two figures (figs. 66, 67), it is seen that the coarse granulations are disappearing, leaving the karyosome standing out plainly, with a more or less distinct clearer space round it. In the last two figures (figs. 68, 69), which represent the last stage of extraction and the condition that is most frequently met with in the preparation, the trophonucleus is reduced to an oval space of faint pink colour, in which the karyosome or karyosomes stand out sharp and clear—exactly the same state of things which is found in preparation *c* (figs. 10, 11), stained with iron-hæmatoxylin, and which results also from other stains, such as methyl-green, Twort's stain, etc. The resemblance between preparation *a*, when fully extracted, and preparation *c*, extends also to other points. The flagellum is fine and delicate in structure, the blepharoplast minute and scarcely visible, and the periplast-line stands out sharply on the concave sides of the body. But there is one remarkable point which puzzled me greatly, and which I took great pains to assure myself was real and not due to errors in representation, namely, that in preparation *a* (figs. 64–69), the trypanosomes are constantly smaller as a whole than in preparation *b* (figs. 62, 63). This is difficult to explain. It may be that the preparation *b* has dried slightly during the process of fixation, and so resisted the tendency to shrinkage, which I believe must always be reckoned with in preparations mounted without drying in Canada-balsam. But this is evidently not the whole of the explanation, for the fully extracted trypanosomes in preparation *a* (figs. 68, 69) are smaller than those in *c* (figs. 10, 11), stained with iron-hæmatoxylin. It seems to me quite possible that when a considerable quantity of stain deposited in the body of the trypanosome is dissolved out, the body may shrink to some extent as the substance of the stain is removed. I do not know how else to explain the distinctly smaller size of the trypanosomes in question.

There is one further point relating to the nucleus which I find very difficult to explain, namely, the frequent appearance, in trypanosomes stained with Giemsa's stain in the ordinary way, of a lighter spot at or near the centre (figs. 61, 70, 80, 81, etc.). From a comparison with preparations in which the stain is more or less extracted, this clear spot appears to correspond to a space or chromatin-free area close beside the slightly excentric karyosome; it does not appear, however, to be always present.

I pass to the consideration of the action of the Romanowsky stain on the flagellum and periplast. It is well known that both these structures stain red with the stain. Schaudinn first, and after him Prowazek and others, have attributed this to a similarity in origin and substance between the locomotor apparatus and the nucleus. According to Schaudinn (1904, p. 395) the entire locomotor apparatus (Trypanosoma noctuæ) is a nuclear product, and the periplast stains red like the nucleus, because it contains myonemes originating from the mantle-fibres of a nuclear spindle. Since Schaudinn wrote, the staining properties of the flagellum have generally been considered as due to its affinities with the chromatin of the nucleus. This is a good instance of the danger of founding conclusions of this sort on the results obtained by a single staining method; for when trypanosomes are stained with Twort's combination of neutral red and Licht-grün, the flagellum, blepharoplast and periplast stain green, in sharpest contrast to the two nuclei, which are both stained red (Pl. 23); hence this method leads to conclusions diametrically opposite to those that can be drawn from the results given by the Romanowsky combination.

I cannot claim to have an expert knowledge of the chemical changes that go on, and the staining substances that result from them in the Romanowsky mixture; but certain conclusions seem to me legitimate, and, indeed, quite obvious. In the first place the mixture contains eosin, or its equivalent, erythrosin. Eosin is not, however, a stain for chromatin, and it cannot be held accountable for the red coloration of the chromatin. When trypanosomes are stained with methylene blue and eosin by Chenzinsky's method, the chromatin stains a pure blue colour. It is generally acknowledged that there is, in the Romanowsky combination, a red stain other than eosin at work which is accountable for the red stain of the chromatin. If it be true, however, that in the Romanowsky mixture at least two red stains are present, the one a stain for chromatin, the other not, it is evident that the fact of two structures being stained red by the mixture is no proof whatever of any similarity in nature or substance between them. Hence the red stain of the flagellum and periplast is not proof in itself of their relationship to chromatin.

(2) Heidenhain's iron-hæmatoxylin.—For this stain I make use of $3\frac{1}{2}$ per cent. solution of iron-alum in distilled water and $\frac{1}{2}$ per cent. solution of hæmatoxylin, which is made up as follows: A stock solution is made in the proportions of 1 gramme hæmatoxylin, 10 c.c. absolute alcohol, 90 c.c. distilled water; for use the stock solution is mixed with an equal volume of distilled water.

In agreement with Schaudinn (1902, p. 190), I found it necessary to let the mordant and stain act for a long time. My procedure is as follows: Films, however fixed, are brought into absolute alcohol and kept there for an hour or so, then brought down through a series of grades of alcohol, differing by 10 per cent. between two consecutive grades, into distilled water, thence into the iron-alum solution, in which they are left till the next morning. The films are then dipped for an instant into distilled water, transferred to the hæmatoxylin solution, and left for at least twenty-four hours. I use both the iron-alum and hæmatoxylin solution in the solid watch-glasses mentioned above, taking care that none of the solution, in either case, gets on to the clean upper surface of the coverslip.

The important part of the whole method is the process of extraction of the stain. After being in the hæmatoxylin the film is washed with distilled water and placed in the iron-alum solution; in a short time clouds of colour can be seen coming out, especially if the coverslip be gently moved; it is then taken from the iron-alum and placed in tap-water, which stops the process of extraction. I now examine the film, in tap-water in a watch-glass, with a dry lens (Zeiss D with oc. 4). If the karyosomes of the trypanosomes can be seen clearly the extraction is sufficient, if not the film is put back again into the iron-alum for a short time, and the process of extraction and examination repeated. When the extraction is judged to be sufficient, the films are washed for twenty minutes in a current of tap-water, which is easily done by letting them float on the surface of the water in a beaker through which a gentle current of water is passed from a pipe immersed below the surface of the water; the cover-slip will neither sink nor run over the edge of the beaker with the current, and can be washed with safety for any length of time. After the tap-water I put them for a few minutes in distilled water, then pass them up through the grades of alcohol into absolute, in which I leave them for at least an hour, then pass them through xylol into Canada-balsam.

As a rule I have always done several films at the same time, and extract the stain to different degrees in different films (compare figs. 25–28, 29–32). When the trypanosomes come out of the hæmatoxylin they appear uniformly black all through. The stain first comes out of the general cytoplasm; next out of the cytoplasmic granules and the body of the nucleus, leaving the karyosome sharp; with more extraction it is taken out of the flagellum and blepharoplast; the bodies that retain it the longest are the kinetonucleus and karyosome, especially the former. In fish-trypanosomes I was able, in some instances, to see myonemes after moderate extraction when the stain was only removed from the general cytoplasm; but in Trypanosoma lewisi I have never succeeded in seeing myonemes.

Iron-hæmatoxylin is the stain of choice for minute structural details of the nuclei and locomotor apparatus. Perfect reliance can, in my opinion, be placed on the results yielded by it, and the stain can always be trusted to give uniform results with the same degree of extraction. It stains most sharply and clearly after sublimate mixtures, less so after osmic vapour and alcohol simply. I have never had good results with it in films dried at any time, before or after fixation. I have also never found any advantage, so far as trypanosomes are concerned, in counter-staining with eosin, orange, etc.; all such processes appear to me simply a waste of time.

(3) Twort's stain.—This is a combination of neutral red and Licht-grün invented by Dr. Twort, of the Bacteriological Laboratory, London Hospital Medical College, who has

kindly furnished me with the following directions for making up and using the stain :

"To prepare the stain make up half-saturated watery solutions of neutral red and of Grübler's light green, using distilled water. Place the neutral red solution in a large open vessel, and add to it sufficient light green solution to combine with the neutral red; the compound will form a precipitate. It is better not to have an excess of either stain, as the precipitate is difficult to wash. When the neutralisation-point is reached, the water will contain a small quantity of both dyes in solution, giving it a dark appearance; the presence of both stains in solution can be easily tested by dropping a drop of the filtrate on to blotting paper; this will spread out, leaving a light red central zone and a faint green outer zone.

"To collect the precipitate it is better not to filter, for the stain soon blocks the pores of the filter-paper. If the solutions are warmed to about 30° or 40° C. before mixing, the precipitate will form in large sticky masses, some of which containing air-bubbles float to the surface and can be removed with a spatula. The rest settle and stick to the bottom and sides of the vessel, and can be collected after pouring off the fluid. The precipitate so collected is rinsed in distilled water and dried at 37° C. In this state it forms dark greenish masses, insoluble in water, somewhat soluble in ethyl alcohol, but soluble to a greater extent in methyl alcohol, and especially so if 5 per cent. of glycerine is added.

"To make up the stain for use, it is best to pound up about 0·25 grm. of the stain with some clean, sharp sand; this prevents the stain going into a sticky mass when the alcohol is added. To the powder so obtained is now added some purest methyl alcohol, acetone-free, containing 5 per cent. by volume of glycerine. Pound up well to obtain a saturated solution; then pour off and add a further quantity of alcohol glycerine solution and repeat the pounding; about 100 c.c. stain can be made from the quantity given.

"The alcohol-glycerine mixture dissolves about ·1 per cent.

of the stain, but it is always better to work with an excess of the powder when grinding up, otherwise it is very difficult to obtain a saturated solution.

"The solution when filtered should be kept in a good-stoppered bottle (and if a completely saturated solution has been obtained add 10 per cent. more alcohol-glycerine mixture).

"Stain for five or ten minutes with the stain made up by mixing one part of distilled water with two parts of the glycerine-alcohol stain-solution. Rinse in distilled water. Fix for half to one minute in Unna's glycerine-ether mixture, 2 per cent. in distilled water. Rinse in distilled water.

"Differentiate and dehydrate in absolute alcohol. Should there be much precipitate this can easily be removed by a few drops of methyl alcohol or equal parts of absolute alcohol and xylol. Remove absolute alcohol with xylol and mount in Canada-balsam in the usual way."

I made a number of smears of blood containing T. lewisi in order to try the effects of Twort's stain, and also used the stain mixed in different proportions with distilled water, and allowed to act for varying lengths of time. I could not obtain good results with any smears fixed with osmic acid, either when smears previously exposed to osmic vapour were subsequently treated with absolute alcohol or sublimate-acetic or when smears were fixed direct in Flemming's or Hermann's fluids. On the other hand I got excellent results with smears fixed direct in sublimate mixtures, either sublimate-acetic, Schaudinn's fluid, or Mann's picro-corrosive, with or without formol (Pl. 23). As regards the application of the stain I did not obtain good results with weak mixtures used for a long time, but I got my best results by mixing the stain in equal quantity with distilled water, or by using two or even three parts of stain to one of distilled water and allowing the stain to act for from twenty minutes to an hour. I differentiated with 5 per cent. Unna's glycerine-ether solution. I found that when the staining mixture was placed on the slide or coverslip a very dense precipitate formed on the smear, but by placing the stain in a suitable vessel such as a watch-glass, and putting the slide or coverslip in it with the smear downwards, no precipitate was formed on the smear.

My recommendations for the use of this stain for trypanosomes are, therefore: fix with sublimate mixtures; use the stain strong, 50–75 per cent.; stain for an hour, more or less; and place the smear downwards in the stain.

Twort's stain, for "show" preparations, has the fault that it stains rather faintly; this might, perhaps, be overcome by letting it act longer. On the other hand it gives results which are extremely instructive and important (Pl. 23). The trypanosomes are stained in two colours, red and green. The two nuclei and the chromatoid granules are red; the flagellum, blepharoplast, and periplast are green. The general protoplasm appears to have a greenish tint, perhaps due to the periplast. The structure of the trophonucleus and the size of the kinetonucleus are just as they are after iron-hæmatoxylin.

Other stains.—I made trial of a number of other stains, or combinations of stains, none of which gave results which could permit me to recommend their use for "show" preparations, but in some cases the results are interesting as reactions.

Delafield's hæmatoxylin.—Stock-solution made up as recommended in Bolles Lee's 'Vade-Mecum' (5th edition, p. 184). For staining, a few drops added to about 30 c.c. distilled water, acidulated with one drop of glacial acetic acid; films stained in it for several hours, then washed with distilled water, brought up through the grades of alcohol into 90 per cent.; left in this overnight, then mounted in the usual way.

Effects (after Hermann's fluid) very poor; the trophonucleus a narrow dark patch with a clear space round (effect of fixative, see above); kinetonucleus indistinct; flagellum faintly stained; cystoplasmic granules rather deeply stained, giving the body a blotchy, marbled appearance.

I think it highly probable that Delafield's hæmatoxylin would have given better results after other fixatives. I have recently obtained excellent results with it, on other material, after fixation with Schaudinn's fluid and Mann's fluid; but as the preparations stained with it are so inferior to those obtained with iron-hæmatoxylin, it did not seem to me worth while to waste time on experimenting with it.

Gentian violet and safranin.—I used these two stains made up as follows: 1 grm. of the stain in 90 c.c. aniline water with 10 c.c. absolute alcohol (Hermann's formula).

Films fixed in Hermann's fluid or Schaudinn's fluid, and stained in gentian violet for half an hour or so, were washed with absolute alcohol, then extracted in absolute alcohol saturated with orange G, then mounted in Canada-balsam after passage through xylol.

Result (fig. 40): Trophonucleus a dark patch with karyosome not distinct; kinetonucleus indistinct; flagellum faintly stained; cytoplasm showing the very same appearance as after Delafield's hæmatoxylin.

Other films, fixed as before, were stained in safranin at least twenty-four hours, washed with absolute alcohol, acidulated alcohol, and absolute alcohol again in rapid succession, then stained in gentian violet and differentiated with orange as already described.

The only effect (figs. 41, 42) produced by the additional treatment with the safranin was to deepen the staining of the cytoplasmic granules, which assume a brownish, muddy tint, and so increase the marbled, blotchy appearance of the cytoplasm.

Methylene blue and eosin.—Used according to Chenzinsky's method, as given in Bolles Lee's 'Vade-mecum' (5th edition, p. 222); films fixed with Hermann's and Schaudinn's fluids. Grübler's methylene blue BX was used.

Result: Exceedingly poor as show preparations, but interesting as showing the two nuclei and the cytoplasmic granules stained blue; no other parts of the body are stained at all, hence the trypanosomes appear shadowy and ghost-like, recognisable at first only by their nuclei and granules, but after careful examination the other parts of the body can be made out. The trophonucleus appears as a blue patch, with the karyosome not very distinct; the kinetonucleus is sharp and deeply stained, its size the same as after iron-hæmatoxylin. The body shows blotchy blue granules, varying in amount in different specimens.

Carmine stains.—I have tried picrocarmine and other carmine stains on trypanosomes, but have never obtained any results worth mentioning.

One cause of the defective results given by some of the stains mentioned above—for instance, Delafield's hæmatoxylin, safranin, gentian violet—seems to me to be in the fact the stain colours the cytoplasmic granules so deeply as to obscure other parts, especially the trophonucleus, which stains less deeply.

It must, I think, be the experience of every one who has tried different methods of technique on different objects, as it certainly has been my experience, how impossible it is to infer or predict the results that a given method will yield for a particular object from the results that it yields on other objects. This truth is constantly brought home to anyone studying trypanosomes, as one so frequently obtains preparations in which the trypanosomes are vile, while the leucocytes are beautifully stained. Thus in films stained with Twort's stain after Hermann's fluid, the leucocytes leave nothing to be desired, while the trypanosomes are useless. Similarly in my preparations stained with methylene-blue-eosin the leucocytes are exquisite.

I have had some experience of the results of technique on another class of objects which might be expected to be not so different from trypanosomes in their reactions, namely, the collar-cells of sponges. A collar-cell is a flagellate organism which might be described as possessing a non-undulating membrane, perhaps similar, morphologically, to that of trypanosomes, but not connected with the flagellum and not contractile in the same manner, only slowly retractile. No à priori reason presents itself to me that would lead me to expect a trypanosome and a collar-cell to be very different in their staining reactions.

Beautiful preparations of collar-cells can be obtained by fixation in osmic acid followed at once by staining with picrocarmine; the same method is absolutely useless for trypanosomes. The osmic-picrocarmine method shows the nucleus of the collar-cells as a deeply stained pink mass, in which a nucleolus can just be made out, but beautiful preparations of nuclear structure in collar-cells can be obtained by fixation in Flemming's or Hermann's fluids followed by Delafield's hæmatoxylin, safranin, gentian violet, methylene-blue-eosin, etc.—just the methods, in fact, which I used on trypanosomes with such ill success (but with excellent results on the leucocytes).

It seems to me, therefore, quite illogical to argue that a method is good or bad for trypanosomes or any other cells, because it is good or bad for some quite distinct class of object. Every kind of cell requires its own special technique, which must be established empirically by trial, and can be discovered only to very limited extent and with great uncertainty by analogy.

Part II.—The Structure of Trypanosoma Lewisi.

(1) General Structure, Form and Dimensions.

As I have stated above, I believe that the preparations fixed simply with osmic acid vapour, and examined in the blood with or without addition of acidulated methyl-green but without further treatment of any kind, represent the nearest possible approach to the living condition in form and dimensions. In such "standard" preparations (figs. 1–8) the body of the trypanosome appears long and slender, sharply marked off from the distinct undulating membrane and flagellum. The kinetonucleus is seen near one extremity, which for convenience may be termed "posterior." The trophonucleus is seen as an oval, well-defined space, containing the distinct karyosome, and situated at rather more than two thirds the length of the body from the posterior end. We may, therefore, for purposes of description, divide the body into three regions: the pre-nuclear region in front of the trophonucleus; the inter-nuclear region between the two nuclei; and the post-nuclear region behind the kinetonucleus.

In the inter-nuclear region the body is roughly cylindrical, but slightly thicker midway between the nuclei. In the pre-nuclear region the body tapers very rapidly to a fine filamentous prolongation, often difficult to distinguish from the flagellum. In the post-nuclear region the body also tapers evenly and rapidly to a point.

The flagellum arises from the blepharoplast or basal granule situated in front of the kinetonucleus, and runs at once slantingly forwards up to the surface of the body to form the marginal flagellum, running along the edge of the undulating membrane and continued beyond the anterior extremity as the free flagellum. The breadth of the undulating membrane appears greater or less, according to the view presented by the trypanosome; so far as can be judged, it is rather more than half the greatest thickness of the body. In this trypanosome the undulating membrane is not much pleated and runs in even, sinuous curves, corresponding to the twists of the body. In any preparation the trypanosomes are found in all sorts of positions, but, speaking generally, the body usually shows one or two main curves, and may be briefly described as either more or less **C**-shaped or **S**-shaped. In either case the undulating membrane keeps always to the convexity of the curves, crossing over the body between each bend. The explanation of this is to be found in the fact that the marginal flagellum is considerably longer than that portion of the body along which its runs, as can be determined easily by actual measurement; it is shown in the table of measurements given below that the average length of the pre-nuclear and inter-nuclear regions together is 19·26 μ, while the average length of the marginal flagellum is 23·51 μ. If we imagine the marginal flagellum as an elastic fibril joined by the undulating membrane to a considerably shorter body, plastic and (after death) inert, we can understand the body being thrown into curves by the elasticity of the flagellum, which, being longer, necessarily runs on the outside of the curves. Only in a single instance have I seen the marginal flagellum on the concave side of a curve in a preparation of T. lewisi (fig. 9), and in this case the flagellum showed a double bend with sharp angles at this point, suggesting a forcible pleating of the flagellum, owing to the fact that the body, wedged in between blood-corpuscles, was unable to take the curvature which the elasticity of the flagellum would naturally cause.

I give in a table below a number of measurements of ten trypanosomes from standard preparations made in the following way. The trypanosomes were first of all carefully drawn with the camera lucida, projected to a magnification of 3000 linear, as determined by substituting for the trypanosomes a stage-micrometer, of which the divisions were drawn in the same manner and the resulting scale measured. The magnification thus obtained depends, other things being constant, on the height of the camera lucida from the drawing board, that is to say, on the length of the tube of the microscope. By trial a length of tube was found, which, with the combination of lenses used, projected the divisions of the micrometer-scale, each representing ·01 mm., to a scale in which they were 30 mm. apart. Taking the magnification of 3000 obtained in this way as accurate, the measurements shown in the table were obtained.

The measurements were carried out by following the undulations of the trypanosomes in the figures with a piece of thread, the length of which was then measured in millimetres; the figure obtained was divided by three, and the resulting figure represents, therefore, the actual length in microns. The pre-nuclear region is measured from the karyosome, or when two nuclei are present (fig. 6) from the boundary between them, to the extreme anterior end of the body; the free flagellum from the latter point to the end. As it is often difficult to determine exactly the anterior termination of the body, these two measurements are a little uncertain and subject to considerable variation. The internuclear region is measured from the karyosome to the centre of the kinetonucleus; the post-nuclear length is from the kinetonucleus to the posterior extremity of the body. The undulating membrane is measured by following up the sinuosities of the marginal flagellum from the kinetonucleus to the anterior extremity of the body.

	Total length (body and free flagellum).	Body.				Flagellum.	
		Pre-nuclear region.	Inter-nuclear region.	Post-nuclear region.	Greatest breadth.	Undulating membrane.	Free flagellum.
(Fig. 1)	28	—	—	—	1·5	21	7
(Fig. 2)	32	—	—	—	2	24	8
(Fig. 3)	30·5	6	12	4·5	2	21	8
(Fig. 4)	28·6	6	11	4	1·6	27·6	7·6
(Fig. 5)	28	8	11·3	3·3	1·6	21·4	5·6
(Fig. 6)	31	7	12·5	5	1·6	24	7
(Fig. 7)	33	6·3	13	5·3	1·5	25	8·3
(Fig. 8)	32	9	12·5	5	1·5	24·6	5·3
	32·3	8	12·5	5	1·5	24	7
	30·3	6	13	4	1·6	22·5	7
Average	30·57	7·04	12·22	4·5	1·64	23·51	6·88

Salvin-Moore, Breinl, and Hindle have recently (1908) published an elaborate study of Trypanosoma lewisi. Since they deal chiefly with the multiplicative period, when the trypanosomes vary enormously in size and form, it is difficult to compare their results with mine. Their fig. 1 appears to represent a so-called "adult" trypanosome, in the period when multiplication is past, comparable to the forms with which I am dealing in this memoir. If that is so, I think anyone who has the most elementary acquaintance with T. lewisi will agree with me that their technique has distorted and deformed the creature very greatly. This is not surprising to me, since these authors rely on Flemming's fluid as a fixative, and I have always found this mixture to give the worst results of any that I have tried as regards the general form of the body (compare my figs. 36–38).

It does not, however, follow that because the external form is distorted the internal structure is necessarily falsified. I have got the sharpest differentiation and clearest pictures of the cytological details in preparations fixed with sublimate mixtures, in which the body as a whole is undoubtedly diminished in size. But I do not think that the technique of Salvin-Moore and Breinl (1907) gives them any right to deny the very obvious trimorphism seen in T. gambiense, for instance, since their preparations, to judge by their illustrations, are obviously inferior to the ordinary osmic-vapour Romanowsky preparations for a study of the form, size, and general structure of the body. I have elsewhere contested their statement that T. gambiense shows no form-differentiation. T. lewisi, on the other hand, is certainly remarkably uniform in structure during the non-multiplicative period. It is not possible to distinguish any distinct types of form, nor anything but slight variations in size, as shown in my table above. The differences that can be observed in the kinetonuclei of different forms are dealt with below.

(2) The Periplast.

That trypanosomes have a fairly strong and resistant membrane or cuticle at the surface of the body is obvious from the manner in which they preserve their body-form under trying circumstances. This fact is brought home to anyone who has studied the trypanosomes and trypanoplasms of fishes. As I have shown elsewhere,[1] in smears of fish-blood dried before fixation the trypanosomes may be almost perfect in form and appearance, while the trypanoplasms side by side with them in the same preparations are deformed almost beyond recognition. This alone is a sure indication that the body-cuticle, commonly termed periplast, is very delicate in the trypanoplasms but comparatively strong in the trypanosomes.

[1] 'Proc. Zool. Soc.,' 1909, pp. 3—31, Pls. I—V.

In Trypanosoma lewisi it is not difficult to discover the existence of a fairly thick periplast, which can be stained and shown up by a variety of methods. After the Romanowsky stain it is usually seen at the edge of the body as a red line, most distinct as a rule on the side opposite to the flagellum, where sometimes it is almost as deeply stained as the flagellum itself (figs. 80, 81). With Twort's stain the periplast stains green and is often very distinct (Pl. 23). With iron-hæmatoxylin the periplast stains very faintly and appears excessively delicate.

In many preparations the periplast becomes very distinct, not by being coloured itself, but by the layer of the body immediately under it becoming clear and transparent, so that the periplast appears, on the side opposite to the flagellum, to stand out from the body as a delicate line, very easily overlooked in preparations in which it is not stained. Figs. 64–69 show very well the genesis of this periplast-line in the process of extraction of Giemsa's stain. It is also seen well in the preparations fixed with Schaudinn's fluid followed by Twort's stain (fig. 85), which colours the periplast green, as already stated.

In preparations stained with Romanowsky stain, after being preserved in various ways, and either dried off or mounted in Canada-balsam without ever being dried, some trypanosomes may be found showing creases and folds of the periplast, which are stained red and simulate fibrils running more or less longitudinally (see especially figs. 70–72). I shall return to this point again later on.

In or immediately under the periplast there are found in many species of trypanosomes, as is well known, distinct contractile fibrils or myonemes. I have myself seen very clearly, and described elsewhere, myonemes in Trypanosoma percæ; less distinctly in Trypanosoma granulosum. I found them most clearly shown in preparations fixed first with osmic vapour, then with Schaudinn's fluid, and stained with iron-hæmatoxylin, very slightly extracted.

In T. lewisi I have not succeeded in seeing myonemes, in spite of much searching of preparations fixed and stained in various ways. I do not know whether my failure to find them is due to the minuteness of the object, or to my not having hit off just the right degree of extraction of the iron-hæmatoxylin stain. The trypanosomes in which I have seen myonemes have been very large species, and it is possible that in T. lewisi the myonemes are too minute to be resolved with the magnification used. On the other hand, myonemes appear to give up the stain very readily, while if over-stained they are obscured by the darkness of the preparation. There can be hardly any doubt that these active flexible organisms must possess a contractile apparatus which suitable methods of technique or optics would reveal.

(3) The Cytoplasm.

In the thickest part of the body, that is to say, in the inter-nuclear region and immediately in front of the trophonucleus, the cytoplasm shows commonly in preparations two distinct regions, a peripheral zone, situated immediately below the periplast, and an axial portion.

The peripheral zone is usually seen in the ordinary Romanowsky preparations as a more or less distinct border marked off by its red tint from the axial bluish region (figs. 60, 61). When the stain is extracted, however, the peripheral zone becomes clear and apparently empty, leaving the periplast-line standing out in the manner already described (figs. 62–69). It is quite evident, from a comparison of different specimens, especially as regards breadth, that the appearance of the periplast-line is really due to the clearing up of the region immediately below the periplast and not to a dilatation of the periplast, or artificial raising up of it from the body. The question is, How is the clearing up of the region under the periplast effected ? Is it a clear zone of protoplasm which takes the red stain, and from which the stain is extracted, or is it really an empty space in which the stain is deposited and from which it is dissolved out again ? And if it is an empty space is it one naturally present, filled only with fluid in the living condition, or is it produced artificially by shrinkage and contraction of the body-cytoplasm? These are difficult questions to answer, but I think that, so far as the trypanosomes in the preparations are concerned, the clear zone is shown to be really a space: first, by a comparison of the trypanosomes fixed by certain methods, for example, Mann's fluid (figs. 29–35, 77–79, 86, 87), in which a comparison with other preparations shows clearly that the thinness of the body is due to an obliteration of the clear area under the periplast by shrinkage, so that the periplast comes into contact with the axial portion of the cytoplasm; secondly, from the frequent creases and folds in the periplast already mentioned, indicating the existence of a space under it which is either empty or at least not completely filled out.

Since, on the other hand, no trace of a clearer peripheral zone can be seen in the living trypanosome nor in the standard preparations, I am inclined to think that the appearance of the peripheral clear zone under the periplast is due simply to shrinkage of the cytoplasm in preparations, leaving a space at the periphery in which the red stain of the Romanowsky combination becomes deposited. This conclusion may be supported also on general grounds; it is common in protozoa to find a more fluid endoplasm surrounded by a less fluid ectoplasm. It is, on the other hand, very unusual to find the peripheral region of the cytoplasm of more fluid nature than the region situated more internally.

For all these reasons I am of opinion that the clear space seen under the periplast is an artefact, the result of shrinkage of the cytoplasm produced by the processes of dehydration and clearing, necessary when never-dried preparations are mounted in Canada-balsam. No such space is ever found in dried-off preparations.

Apart from the nuclear and locomotor apparatus, the cytoplasm contains various enclosures. First in importance are certain granules situated chiefly in the inter-nuclear region; as it is convenient to employ a distinctive term for them, I propose to use the term chromatoid grains.[1]

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[1] Compare Woodcock, 'Quart. Journ. Micros. Sci.,' vol. 50, p. 229.

These bodies are best seen after Twort's stain, which colours them red in the midst of the greenish cytoplasm (figs. 82–87). They are also well seen after iron-hæmatoxylin if the stain be but little extracted (figs. 28–30), and are most distinct when the trophonucleus appears as an evenly stained black patch without any detail; if, however, the extraction be carried further the stain comes out of them, and in preparations in which the karyosome stands out sharply from the trophonucleus the chromatoid grains are no longer visible (figs. 14, 15, 25, 31, 32). With the Romanowsky stain the chromatoid grains can seldom be made out; sometimes (figs. 61, 74, 80) a few red granules are to be made out, but more usually the cytoplasm stains an even bluish or purplish tint, which may mean either that the chromatoid grains are stained red, but are obscured by the dense blue colour of the general cytoplasm, or that they also take the blue coloration of the Romanowsky stain. After Delafield's hæmatoxylin the chromatoid grains stain a dull violet like the nucleus; after gentian-violet-orange and safranin-gentian-orange they also stain like the nucleus; after methylene-blue-eosin they take the blue colour. With all these stains, however, and also with iron-hæmatoxylin, the general cytoplasm is tinged in the same manner, probably owing to the presence of minute granulations diffused through it which also take up the stain. Hence the chromatoid grains do not appear after the five staining methods mentioned as definite sharply marked bodies, but as blotchy, ill-defined patches which give the cytoplasm a marbled appearance.

Twort's stain alone, of all I have tried, differentiates the chromatoid grains clearly from the surrounding protoplasm. By this method they appear as coarse granules stained a faint reddish tinge, irregular in form and not sharply contoured. They vary in amount, being sometimes spread over the whole inter-nuclear region, and even extending into the pre-nuclear region; in other cases there are only a few of them, occurring chiefly just behind the trophonucleus (figs. 82–87). These variations in the quantity of the chromatoid grains do not appear to be correlated with any other structural variations of the trypanosome.

By their reactions to the stains mentioned, the chromatoid grains are evidently allied to the chromatin-elements of the nuclei; they are stained in a manner similar to the trophonucleus by hæmatoxylin, gentian-violet, safranin, methylene-blue, and neutral red (in Twort's stain). The Romanowsky stain alone fails to differentiate them clearly, but I have said enough already in support of my opinion that this stain is quite unreliable as a test for nuclear structures. Comparing the chromatoid grains with the constituents of the trophonucleus (see below), it is clear that their reactions agree very closely with the intra-nuclear chromatin. In preparations in which the stain is extracted from the intra-nuclear chromatin, leaving the karyosome sharp, it is also extracted from the chromatoid grains. When the intra-nuclear chromatin is coloured, the chromatoid grains are also coloured, and to about the same tint. On these grounds I infer that the chromatoid grains represent extra-nuclear chromatin or chromidia, derived from the trophonucleus, possibly from the karyosome (see below).

In ordinary preparations the cytoplasm does not contain any other enclosures than the chromatoid grains. In my standard preparations, however, examined in the wet blood, I find a body which is not to be made out in trypanosomes examined in Canada-balsam or cedar oil (figs. 1–8). Quite constantly a refringent granule is seen, sharply and distinctly, in the post-nuclear region, sometimes close behind the kinetonucleus, sometimes nearer the pointed posterior termination of the body. It has the usual appearance of a refringent granule appearing as a black dot at the lower focus and as a clear spot at the higher focus. It is more refringent and much more distinct than the unstained kinetonucleus. At first I thought it might be Prowazek's "anchoring granule," but I do not find anything anchored to it, and it disappears in the stained and mounted preparation. This is probably due to the fact that the granule itself does not stain, and is only visible by its refringence, and consequently disappears when mounted in refractive media. As to the nature or function of the granule, I am not able to offer any suggestions.

In many trypanosomes in the standard preparations I find a distinct clear space or vacuole immediately in front of the kinetonucleus, varying in size, frequently absent, sometimes double (figs. 5–8). This is perhaps an artefact or post-mortem change, as I have not been able to see it in the living animal, nor does it occur in the ordinary stained preparation.

(4) Locomotor Apparatus.

Under this head are comprised the flagellum with its basal granule or blepharoplast, and the undulating membrane. I have already described above the general configuration of the flagellum and undulating membrane, and shall deal here only with points of minute structure.

The blepharoplast is situated very close to the kinetonucleus, apparently touching it or even overlapping it; that is at least the position which it has in the standard preparations (figs. 1–8) and in all preparations which have been mounted without ever having been dried (compare figs. 62–69). On the other hand, in preparations which have been dried off, either before or after fixation, a considerable interval usually separates the kinetonucleus and blepharoplast (figs. 60, 61, 70, 73, 80, 81), a state of things which must be regarded as artificial. In view of the alleged purity of the technique of Salvin-Moore, Breinl, and Hindle (1908), I am surprised to notice such a condition very frequently in their figures.

In preparations stained with iron-hæmatoxylin or other sharp nuclear stains the blepharoplast appears as a minute granule, a slight dilatation, often hardly visible, of the proximal end of the flagellum (figs. 25–32, etc.). After Twort's stain it is coloured green, like the flagellum itself, in sharp contrast with the nuclei, which are red (figs. 82–87). After the Romanowsky stain the blepharoplast is coloured red, like the flagellum and the nuclei, and instead of appearing as a very minute dot, it is usually considerably enlarged in size, appearing sometimes as a relatively large, diffuse patch (figs. 70, 73). I regard this appearance of the blepharoplast simply as another instance of the tendency of the Romanowsky stain to form concretions, as it were, round the bodies for which has the affinity, whatever it may be, which is shown by staining.

The flagellum presents itself as a delicate filament of even thickness and like appearance throughout its whole length (figs. 10, 11, etc). I have not been able to see any structural differences in it in different parts. After the Romanowsky stain the flagellum commonly appears much thicker and quite coarse in structure, a state of things evidently due to the usual performances of this staining method. When, however, the Romanowsky stain is suitably extracted, the flagellum comes down to its normal thickness (figs. 67–69).

Prowazek (1905) has introduced a most extraordinary complication of fibrils into the structure of T. lewisi (see his text-fig. 2B, p. 359). One fibril is supposed to connect the karyosome with the kinetonucleus; from the latter another fibril runs to an "anchoring grain" situated in the post-nuclear region, and from this grain yet another fibril runs forward to the anterior end of the body. I have searched in vain in all my preparations, without finding a trace of this fibrillar system. In my smears stained with the Romanowsky stain, however, I find that the periplast, stained red, shows creases and folds often closely simulating distinct fibrils. I have represented some of these in figs. 70–72 (Pl. 22), and could have drawn many more. I have a strong suspicion that Prowazek's fibrillar system is founded on nothing but accidental creases of this kind, since he remarks, "Die Fasern selbst lassen sich färberisch nur schwer darstellen und nur an weniger Objekten konnte man sie streckenweise als rote Fibrillen verfolgen" (loc. cit., p. 358). This so exactly describes what I have seen that I must express my profound scepticism as to the existence of Prowazek's fibrillar system.

(5) The Nuclear Apparatus.

Under this head I include simply the two nuclei, trophonucleus and kinetonucleus. I will begin my account with the second of these two bodies.

The kinetonucleus is easily made out in the living condition or in the standard preparations as a refringent body of fair size a short way from the hinder end. It is also seen, especially in the fixed preparations, that there are two types of form presented by the kinetonucleus, which may be either rod-shaped or circular in contour. Transitions occur, but not commonly, between these two types.

With iron-hæmatoxylin the kinetonucleus stains black; the rod-like type stains much more darkly than the round form, which appears of a pale greyish tint when the stain is well extracted; it is then not so dark in tint as the blepharoplast, which is seen as a black dot on the edge of the kinetonucleus (figs. 31, 33). The rod-shaped type of kinetonucleus, on the other hand, takes the stain more darkly than the blepharoplast which is seen close beside it, not overlapping it (figs. 32, 34).

By all nuclear stains the kinetonucleus is stained intensely, more so than any other part of the body, in fact it may be the only part of the body visible in the preparation. After methylene-blue-eosin it is blue; after Twort's stain, red; after the Romanowsky stain, as is well known, it stains a deep purple-red; what is not so generally known is that it comes out, as I have already pointed out, four or five times its natural size (compare figs. 62, 63, with figs. 10, 11). When, however, the Romanowsky stain is suitably extracted, the kinetonucleus comes down to the size that it appears in other preparations (figs. 66, 69, etc.).

I have not been able to make out any structure in the kinetonucleus. It appears to consist simply of a dense mass of chromatin, which is carried on, or impregnates, a basal substance, for which the name "plastin" is in common use.

With regard to the two forms of the kinetonucleus, it is possible that these may be simply two views of the same thing, a body having the form of a disc which, seen edgeways, would appear rod-shaped, and in surface view would present a circular contour. This explanation is compatible with the facts stated above, namely that the rod-shaped type is darker in colour, that there are transitions between the two forms, and that the blepharoplast overlaps the round form, but is found close beside the rod-shaped form. I have not been able to convince myself that this explanation is either true or false.

Salvin-Moore, Breinl, and Hindle (1908) are of opinion that the kinetonucleus is to be regarded as of the nature of a centrosome or blepharoplast, and use the term "end-bead of the flagellum" for the granule termed by me the blepharoplast. The question is one that must be decided by developmental data; a true centrosome is pre-eminently a body of dynamic rather than static function. I do not desire, therefore, to argue the question in this memoir, in which I purposely avoid dealing with division-stages. I will only say that in refusing to allow the kinetonucleus the status of a true nucleus, the authors are ignoring a great deal of work by Schaudinn and others in support of this view. As regards the "end-bead," I am still of opinion that it represents a true centrosome or blepharoplast. In Trypanosoma grayi I found that division of the blepharoplast was invariably the first step in the division of the trypanosomes.

The trophonucleus is at first very puzzling, on account of the extraordinary difference in its appearance after the Romanowsky stain, now so much in use, and all other stains. The coarse "Romanowsky splotch" seems at first sight to have nothing in common with the delicate, refined structure seen after iron-hæmatoxylin or Twort's stain (compare figs. 60–63, with figs 10, 11, and 82–87). I have already attempted above an explanation of the vagaries of the Romanowsky stain; my reasons for regarding the results of this stain as departures from the truth are simply founded on the facts—first, that the condition seen after iron-hæmatoxylin is also seen in the standard preparations, in the living condition, and after every other nuclear stain known to be of value as such;

secondly, that when the Romanowsky stain is cautiously extracted the nucleus comes down to a condition similar to that seen after other stains (figs. 68, 69).

The parts of the trophonucleus may be considered under the heads of the "membrane," the "karyosome" or "karyosomes," and the "intra-nuclear chromatin," the latter term being understood to be exclusive of the karyosome.

The membrane appears as a faint but distinct line circumscribing the oval contour of the nucleus. It does not, however, stand out sharply from the contents, nor does it stain a different colour from the rest of the nucleus; it also never shows a double contour. For all these reasons the membrane may be regarded simply as the superficial limit of the intra-nuclear chromatin, condensed to form a more closely knit zone at the surface. In other words, the membrane is not a true nuclear membrane in the sense in which the term is used for Metazoan nuclei.

The karyosome appears usually as a single small grain at or near the centre of the nucleus; this is the commonest condition, found in about 80 to 90 per cent. of the trypanosomes. I regard this, therefore, as the normal state of the nucleus, and will deal with it first, and describe afterwards other conditions. In order to see the karyosome clearly it is necessary to extract the stain thoroughly when staining with iron-hæmatoxylin, otherwise the karyosome is obscured by the granules of the intra-nuclear chromatin. The extraction should be carried on until the karyosome appears as a sharp, clear, and definite body. With Twort's stain no such precautions are necessary, as the intra-nuclear chromatin does not take it up to the same extent, hence this stain is one of the best for showing the karyosome and the nuclear structure generally. In its reactions the karyosome is very similar to the kinetonucleus, but stains less deeply and gives up the stain more readily. I infer from this that the karyosome is, like the kinetonucleus, a body consisting chiefly of chromatin on a basis of plastin, but that the chromatin is much less concentrated and condensed in the karyosome.

The karyosome shows very considerable variations in some of the nuclei. In the first place it varies in size, being usually quite a minute granule, but sometimes very much larger. In the second place it varies in position, and instead of being central or sub-central it may be very excentric in position, even to the extent of being placed quite at one pole of the oval nucleus. Thirdly, the karyosome, usually single, may become double or multiple, apparently through a process of division or disruption. I have described elsewhere[1] such a process of budding on the part of the karyosome in the case of Trypanosoma granulosum, where the large size of the object makes the process easy to follow out in detail. The minuteness of T. lewisi makes it much more difficult to be quite certain what exactly takes place. Appearances can be found, however, which indicate clearly a process of division or budding from the karyosome (fig. 50), leading to the formation of two karyosomes, subequal or markedly unequal in size; the two bodies thus formed travel apart, and may place themselves at opposite poles of the nucleus (fig. 49). One or both of the karyosomes may undergo further disruption, apparently, so that in addition to a principal karyosome there are smaller granules present, not more than four in number in any case that I have observed (figs. 52, 53, 90); I feel, however, considerable doubt whether some of these smaller granules may not be, in most cases, granules of the intra-nuclear chromatin from which the stain has been insufficiently extracted. In my preparations stained with Twort's stain I find deviations from the condition with a single subcentral karyosome excessively rare; I have, however, seen in these preparations the condition with a double karyosome (fig. 86), and with a larger and two smaller karyosomes (fig. 90).

Prowazek (1905, pp. 361–363) has sought to bring the various conditions of the karyosome under three processes, which he terms "autosynthesis," "reduction," and "parthenogenesis." In autosynthesis the karyosome is supposed to divide into two, and each half divides again; two of the four parts are thrown out, the other two parts "copulate," and the nucleus is reconstituted. Equally complicated processes are seen in reduction and parthenogenesis.

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[1] 'Proc. Zool. Soc.,' 1909, pp. 21, 22, pl. V, figs. 78–93.

For my part I am very doubtful if the different appearances seen in the nuclei of T. lewisi will bear the excessive weight of subjective interpretation which Prowazek places upon them. Moreover, Prowazek seems to have based all this theoretical superstructure upon Romanowsky-stained preparations, which, in my opinion, are altogether false and misleading for minute nuclear structure.

All that can be inferred from a comparison of different trypanosomes is that the karyosome sometimes gives off smaller portions; some of these go to the surface of the nucleus, and I think it highly probable that the chromatoid grains described above take origin in this way.

The intra-nuclear chromatin shows different conditions according to the stain used and the degree of extraction of the stain. With iron-hæmatoxylin, for instance, the whole nucleus is at first a dense black patch, owing to the intra-nuclear chromatin being stained so deeply as to obscure completely the karyosome. A similar condition is seen after the Romanowsky stain. As the stain is extracted, the intra-nuclear chromatin gradually becomes lighter in tint, and the karyosome begins to stand out. The extraction of the colour from the intra-nuclear chromatin does not take place uniformly, however, but some of the granules retain the stain longer than others, while some parts become quickly decolorised; there is very often a distinct clear halo round the karyosome (figs. 17, 18, 39). With complete extraction the intra-nuclear substance becomes pale and gives the impression of an empty space, in which the karyosome is suspended (fig. 10, 11). A quite similar and parallel series of appearances is seen when the Romanowsky stain is slowly extracted, as described above, but in this case the granules in the intra-nuclear space are much larger, more of the nature of concretions, than they are after iron-hæmatoxylin. With Twort's stain the intra-nuclear substance appears a pale red tint, and does not show granulations (Pl. 23); with methylene-blue-eosin it is pale blue. In the standard preparations stained with methyl-green no granulations are to be made out in the nucleus, which has simply the appearance of an oval vesicle, in which the distinct karyosome is suspended (figs. 1–8).

From all these various appearances I conclude that the intra-nuclear space contains, probably, in the living condition a more or less fluid nuclear sap, in which chromatin is suspended in the form of particles, perhaps differing in size or in concentration of substance, but for the most part too minute to be resolved with the microscope, except when very deeply stained, or artificially enlarged by the deposit of the stain round them.

In the foregoing paragraphs I have dealt with the minute structure of the trophonucleus; I will now mention some of its variations as a whole. In the first place it varies considerably in size, as may be seen from my figures. There may sometimes, moreover, be two trophonuclei present, flattened against each other and each of relatively small size (figs. 6, 84). This condition, which is found in a very small number of the trypanosomes, does not appear to have anything whatever to do with division, as all other parts of the body are in the normal single condition; I regard it simply as an abnormality. In rare cases trypanosomes are met with having three trophonuclei (fig. 74).

In preparations fixed with Flemming's fluid (figs. 36–38) and Hermann's fluid (figs. 40–44) the trophonucleus often seems to float in a space in the cytoplasm, a condition which must be regarded as an artefact.

In this memoir I have only dealt with the structure of Trypanosoma lewisi in the resting, "adult" stage. It is my hope in a future memoir to extend these studies to the multiplication-period, and possibly to other developmental periods of the life-history.

LISTER INSTITUTE,
January, 1909.

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EXPLANATION OF PLATES 21–23,

Illustrating Mr. E. A. Minchin's paper on "The Structure of Trypanosoma lewisi in Relation to Microscopical Technique."

All the figures represent preparations of Trypanosoma lewisi, drawn with the camera lucida at a magnification of 3000 diameters (see p. 760).

In order to facilitate the comparison of the results obtained by different methods, the figures may be best classified by the dates on which the trypanosomes represented were preserved. All those of a given date were from the same blood, preserved or stained by various methods.

February 24th.—Pl. 21, figs. 14, 15, 19–22, 39; Pl. 22, figs. 60, 61, 70, 73, 80, 81). (The preparations of this date were made on slides; all others, except fig. 74, were made on coverslips.)

March 5th.—Pl. 21, figs. 9–13, 36, 37; Pl. 22, figs. 62–69.

March 16th.—Pl. 21, figs. 23, 24, 40–53.

March 20th.—Pl. 21, figs. 25–34, 54–59; Pl. 22, figs. 76–78.

March 27th.—Pl. 21, figs. 17, 18, 38; Pl. 22, fig. 75.

March 30th.—Pl. 23, figs. 82–84, 88–91.

April 3rd.—Pl. 23, figs. 85–87.

April 6th.—Pl. 21, figs. 1–8, 16, 35; Pl. 22, figs. 71, 72, 79.

The following is a classification of the figures according to the method of fixation employed for the preparations:

Osmic vapour simply: Pl. 21, figs. 1–8.

Osmic vapour followed by absolute alcohol: Pl. 21, figs. 9–11; Pl. 22, figs. 60–69, 70, 71, 72, 74.

Osmic vapour followed by sublimate-acetic (95:5): Pl. 21, figs. 14–18; Pl. 22, figs. 70, 73, 75.

Osmic vapour followed by Schaudinn's fluid: Pl. 21, figs. 23, 24, 45–53.

Osmic vapour followed by Flemming's fluid: Pl. 21, fig. 39; Pl. 22, figs. 80, 81.

Sublimate-acetic (99:1): Pl. 21, figs. 12, 13.

Sublimate-acetic (95:5): Pl. 21, figs. 19–22; Pl. 23, figs. 82–84, 88–91.

Schaudinn's fluid: Pl. 21, figs. 25–28; Pl. 22, fig. 76; Pl. 23, fig. 85.

Mann's fluid without formol: Pl. 21, figs. 29–32; Pl. 23, fig. 86.

Mann's fluid with formol: Pl. 21, figs. 33–35, 54–59; Pl. 22, figs. 77–79; Pl. 23, fig. 87.

Flemming's fluid: Pl. 21, figs. 36–38.

Hermann's fluid: Pl. 21, figs. 40–44.

Figs. 19–22, 39, 60, 61, 70, 73, 74, 80, and 81 are from preparations dried off after fixation or after staining; all others are from never-dried preparations.

In studying the preparations, at least three trypanosomes were drawn from each, in order to take the average of the individual variations, which, though slight, must still be reckoned with, as seen from figs. 1–8 and the table on p. 789. It was, however, neither possible nor desirable to reproduce all these figures. Where only one or two figures are given from a preparation it must be understood that they represent, as far as possible, the average proportions of the trypanosomes in the preparation. Hence such figures as 21, 22, 75, 79, etc., are not to be taken as representing trypanosomes of small size, but as normal individuals showing the shrinkage produced by the method of preparation, and should be compared with others of the same date (see above) prepared in other ways.

PLATE 21.

Figs. 1–8.—"Standard" preparations (see p. 759). 1 and 2 fixed with osmic vapour in a hanging drop; owing to the thickness of the preparation details could not be made out, but only the outline of the body and the flagellum could be drawn. 3 and 4. Fixed with osmic vapour in a hanging drop, then mounted on a slide and sealed up: the trophonucleus with its karyosome, kinetonucleus, and posterior refringent granule are seen. 3 has a rod-shaped, 4 a rounded kinetonucleus.

5–8. Treated as 3 and 4, but with the addition of acidulated methyl-green solution to the preparation. The same details are seen as in the foregoing, with the addition of a vacuole or vacuoles immediately in front of the kinetonucleus, probably to be regarded as artefacts.

With figs. 1–8 compare especially figs. 16, 35, 71, 72, 79, all drawn from preparations made from the same blood at the same time, but preserved in different ways.

Fig. 9.—Outline of a specimen from the same preparation as figs. 62, 63, showing the flagellum passing on the concave side of a body-curve at a point near its origin, and here having a sharp double bend.

Figs. 10, 11.—Osmic vapour, absolute alcohol, iron-hæmatoxylin, never-dried. Note the distinct periplast-line. Compare especially with figs. 12, 13, 36, 37, 62–69, all preserved at the same time and from the same blood.

Figs. 12, 13.—Sublimate acetic (99 : 1); 12, stained Giemsa; 13, stained iron-hæmatoxylin; never-dried. Compare with figs. 10, 11, etc.

Figs. 14, 15.—Osmic vapour followed by sublimate-acetic (95 : 5), stained iron-hæmatoxylin; never-dried. Compare figs. 19–22, 39, 60, 61, 70, 73, 80, 81, all preserved at the same time and from the same blood.

Fig. 16.—Fixation as in the last; stained Giemsa; never-dried. Drawn in outline to show the great diminution of size. Compare figs. 1–8, etc., made from the same blood at the same time. All the trypanosomes in the same preparation and in another treated in the same way at the same time show the same reduction in size.

Figs. 17, 18.—Fixation as in 14–16; stained iron-hæmatoxylin; never-dried. Compare with figs. 38 and 75, both preserved at the same time from the same blood.

Figs. 19–22.—Fixed with sublimate-acetic (95 : 5) direct. 19, 20. Stained iron-hæmatoxylin; never-dried. 21, 22. Stained Giemsa, then dried off, showing extraordinary shrinkage. Compare figs. 14, 15, etc.

Figs. 23, 24.—Osmic vapour, followed by Schaudinn's fluid; stained iron-hæmatoxylin; never-dried. Note the very distinct periplast-line. Compare figs. 40–44. Preserved at the same time from the same blood.

Figs. 25–28.—Fixed with Schaudinn's fluid direct; stained iron-hæmatoxylin; never-dried. 25, much extracted; 26, 27, less; 28, still less extracted. Compare figs. 29–34, 76–78, preserved at the same time from the same blood.

Figs. 29–32.—Fixed with Mann's picrocorrosive without formol; stained iron-hæmatoxylin; never-dried. 29, 30, less, 31, 32, more extracted. Compare figs. 25–28, etc.

Figs. 33, 34.—Fixed with Mann's picrocorrosive with formol; stained iron-hæmatoxylin; moderately extracted; never-dried. Two trypanosomes in the same field showing the relations of kinetonucleus, blepharoplast, and flagellum very sharply. Compare figs. 25–28, etc.

Fig. 35.—Treated as last. Rather more extracted. Compare figs. 1–8, etc.

Figs. 36–38.—Fixed in Flemming's fluid direct; stained iron-hæmatoxylin; never dried. 36, 37, compare with figs. 10, 11, etc. 38, compare with figs. 17, 18, etc.

Fig. 39.—Fixed with osmic vapour followed by Flemming's fluid; stained iron-hæmatoxylin; never-dried. Compare with figs. 14, 15, etc.

Figs. 40–44.—Fixed in Hermann's fluid direct; 40, stained gentian violet and orange; 41, 42, stained safranin, gentian violet, orange; 43, 44, stained iron-hæmatoxylin; never-dried. Compare figs. 23, 24, etc.

Figs. 45–53.—Nuclei of trypanosomes from the same preparation as figs. 23, 24. 45 shows the most usual condition, occurring in about 90 per cent. of the trypanosomes.

Figs. 54–59.—Nuclei of trypanosomes from a companion preparation to that from which figs. 33, 34 were drawn; treated in the same manner, but the stain rather more extracted. 54 represents the most usual condition.

PLATE 22.

All figures drawn from preparations stained with the Romanowsky stain, either Giemsa's method (G.) or azure-erythrosin (A. E.).

Figs. 60, 61.—Fixed with osmic vapour, then dried off; fixed with absolute (G.). Compare with figs. 14, 15, etc.

Figs. 62, 63.—Osmic vapour; absolute alcohol; never-dried (G.). Compare with figs. 10, 11, etc.

Figs. 64–69.—From a companion slide to the foregoing; more extracted with acetone (see pp. 775–777).

Figs. 70–72.—Trypanosomes showing folds of the deeply-stained periplast simulating fibrils (see p. 797). 70, fixed osmic vapour, then dried off; sublimate acetic (95 : 5), stained (G.); from a companion preparation to that from which fig. 73 is drawn. 71, 72, osmic vapour, absolute alcohol (G.); never-dried. Compare figs. 1–8, etc.

Fig. 73.—Osmic vapour, sublimate-acetic (95 : 5), stained (G.); dried off after the osmic vapour. Compare figs. 14, 15, etc.

Fig. 74.—Trypanosome with three trophonuclei; osmic; absolute alcohol (G.); dried off.

Fig. 75.—Osmic vapour. sublimate-acetic (A. E.); never-dried. Compare figs. 17, 18, etc.

Fig. 76.—Schaudinn's fluid direct (A. E.); never dried. Compare figs. 25–34, etc.

Figs. 77, 78.—Mann's picrocorrosive with formol (A. E.); never-dried. Companion preparation to the last.

Fig. 79.—Treatment similar to the last. Compare figs. 1–8, etc.

Figs. 80, 81.—Osmic vapour, Flemming's fluid (G.); dried off. Compare figs. 14, 15, etc.

PLATE 23.

All figures drawn from preparations stained with Twort's stain; never-dried.

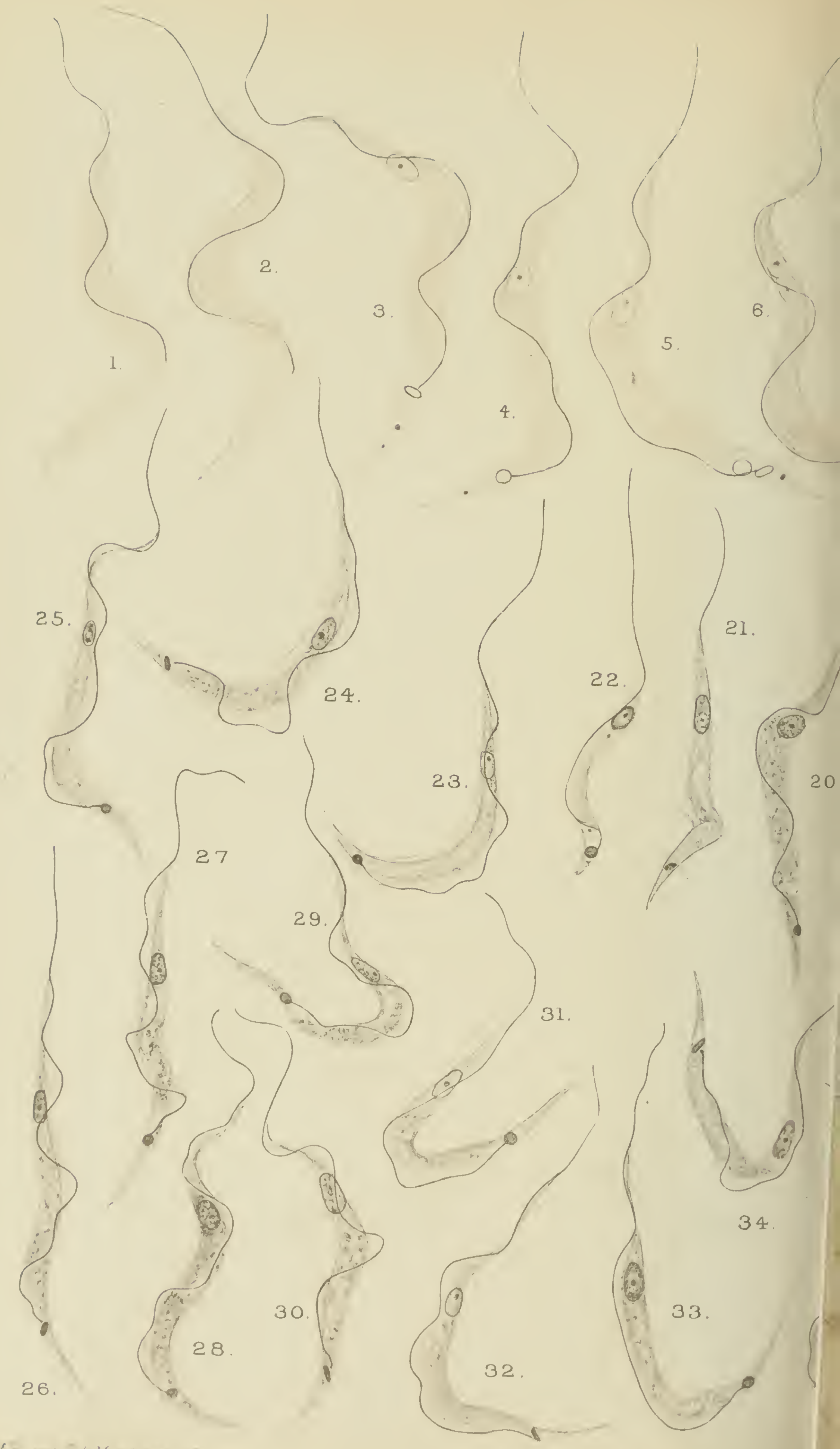
Figs. 82–84.—Fixed in sublimate-acetic (95 : 5).

Fig. 85.—Fixed in Schaudinn's fluid.

Fig. 86.—Fixed in Mann's picrocorrosive without formol.

Fig. 87. –Fixed in Mann's picrocorrosive with formol.

Figs. 88–91.—Nuclei of trypanosomes from the same preparation as figs. 82–84.



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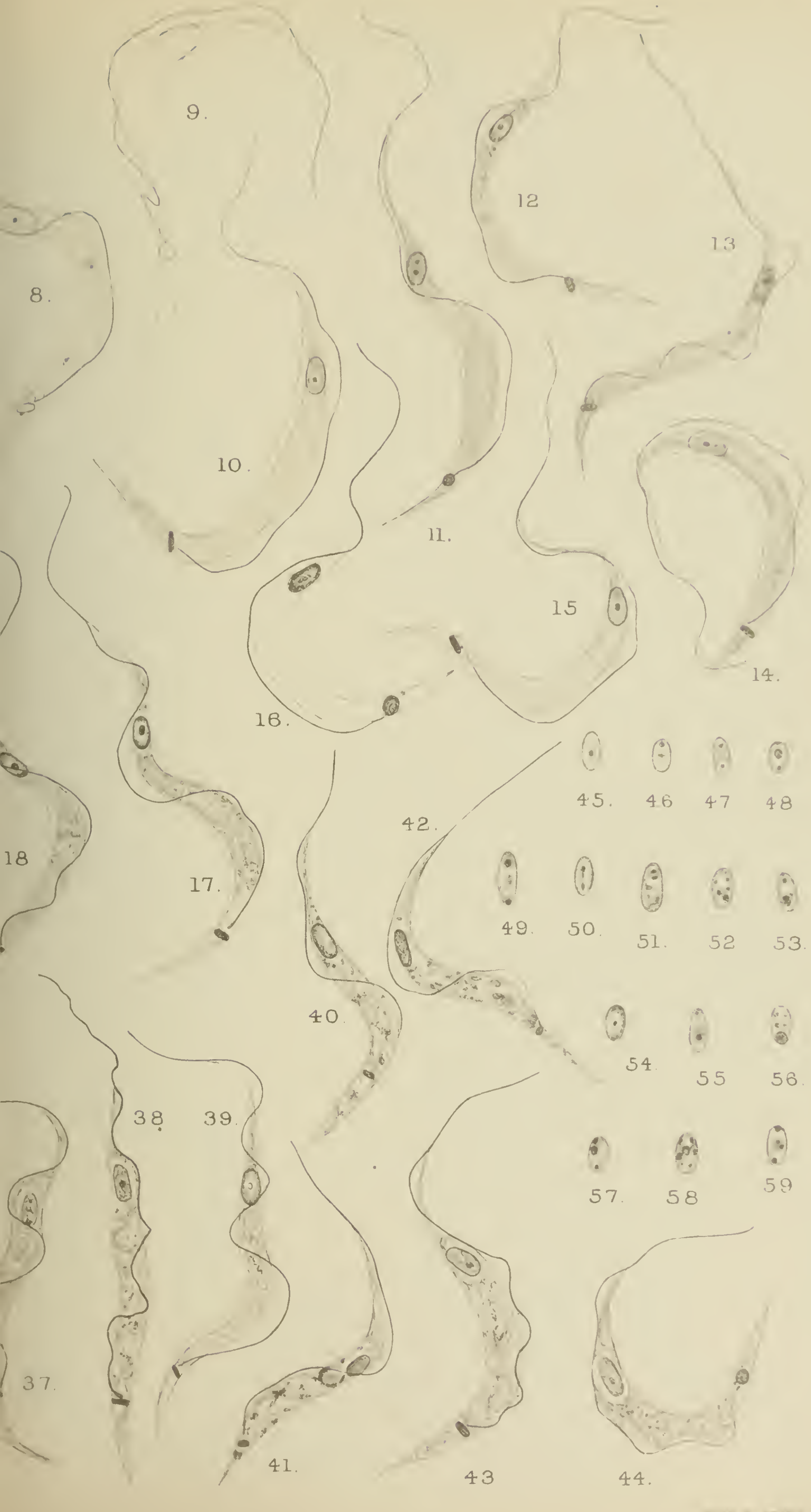
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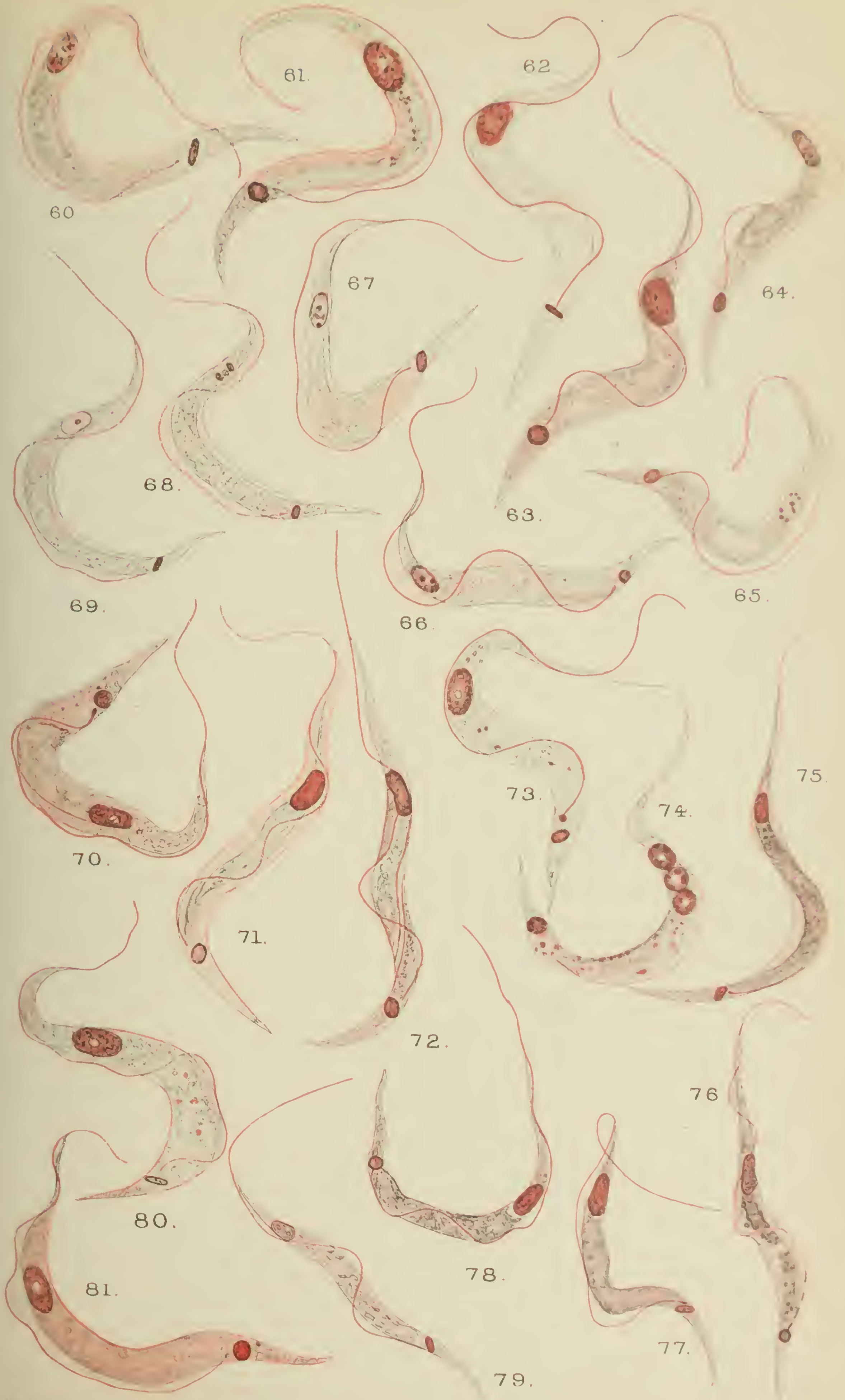

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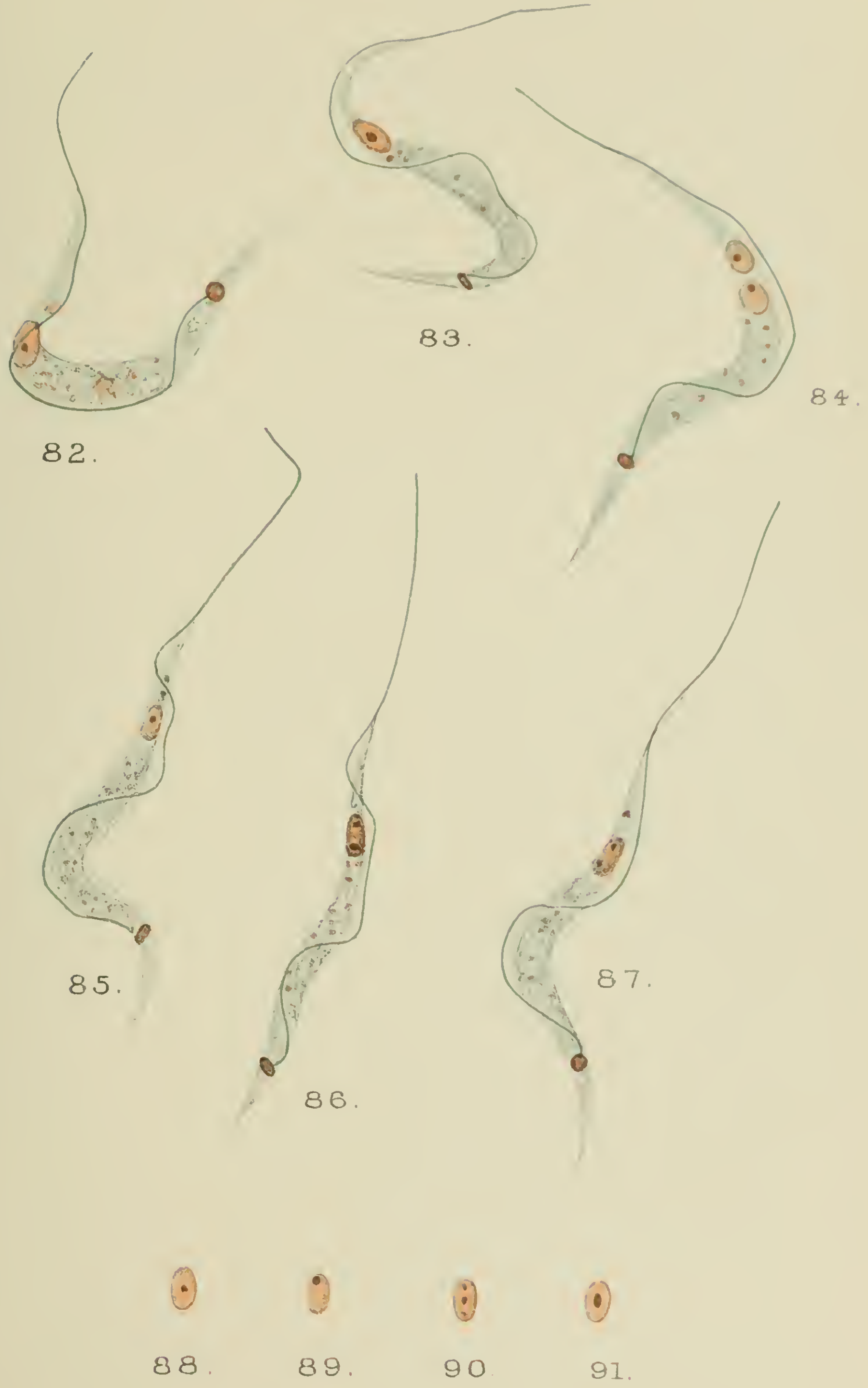

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TRYPANOSOMA LEWISI.

Observations on certain Blood-parasites of Fishes occurring at Rovigno.

By

Prof. E. A. Minchin, M.A., and H. M. Woodcock, D.Sc.

With Plates 8–10.

The objects of this paper are twofold. The first is the description of a hæmogregarine and a trypanosome occurring in a species of Trigla (T. lineata), which is a common gurnard in the Northern Adriatic. We are unaware of any published observations of blood-parasites in this fish, and we regard the hæmogregarine as a new species, for which we propose the name Hæmogregarina rovignensis. Neumann, in his recent account of hæmatozoa of marine fishes at Naples (**15**), mentions the occurrence of a trypanosome which he names T. triglæ in another species of Trigla, namely, T. corax. It is not at all improbable that the trypanosome we have found is the same species as that described by Neumann; we therefore do not propose to give it a new specific name.

The second object that we have in view is the comparison of the minute structure of the nucleus of the above-named hæmogregarine with that of the trophonucleus of a trypanosome. For this purpose, as the trypanosome of the Trigla is very rare in our material, a trypanosome which occurs in much greater abundance in skates (Raia, sp.) in the neighbourhood of Rovigno has been selected. This form is most probably T. raiæ Lav. and Mesn.

With regard to the technique, permanent preparations were made according to both the principal methods in use. Smears on slides were fixed in osmic vapour[1] for half a minute and then placed in absolute alcohol for ten minutes or so. They were stained with Giemsa's solution in the ordinary manner for two and a half to three hours and afterwards differentiated with orange-tannin and acetone. Smears on cover-slips, on the other hand, were at once dropped face downwards into sublimate-acetic mixture or Schaudinn's fluid for fixation, after which they were brought up to 90 per cent. alcohol. These smears were stained either with iron-hæmatoxylin or with Twort's stain and finally mounted in balsam. Fuller details regarding this mode of procedure will be found in Minchin (**11**), the method there described being that followed in the present case. The figures of the hæmogregarine are all drawn to a magnification of 3000, those of the trypanosomes to a magnification of 2000. We are indebted to Miss Rhodes for drawing the greater number of the figures and for colouring nearly all, and take this opportunity of expressing our thanks to her.

I. HÆMOGREGARINA ROVIGNENSIS, N. SP.

(Figs. 1–28, 39–50, and 58–62.)

This parasite was found on three occasions in the early spring. In the three gurnards in which it was seen it was not at all infrequent in the blood, as we have since learnt by the study of stained preparations. The parasite, chiefly in its small intra-cellular form, was observed in the living condition in cover-slip preparations of fresh drops of blood, but only with difficulty, since it was not readily distinguishable from the cytoplasm of the blood-corpuscle. When made out with certainty it was seen as a small oval area, somewhat clearer and paler than the surrounding cytoplasm. No prominent refractile granules were noticed, nor could the nucleus be made out satisfactorily. We did not observe any alteration or movement in these intra-cellular forms so long as they were kept under notice, but in a few of the living preparations one or two small forms were seen free. These in all probability corresponded to the small intra-cellular parasites. They appeared as sausage-shaped bodies very slightly curved. One third or so of the body was granular in character; this probably was the region of the nucleus (cf. description of fixed and stained parasites below). They underwent no active displacement, and it was difficult to feel sure whether they really moved or altered in shape at all or not. Now and again the concavity would appear to lie first on one side of the body and then on the other, and now and then the body would stand on one end, appearing in transverse optical section as a round globule; but these changes were extremely slow, and may have been due to passive rotation of the vermicule, caused by very delicate currents in the blood-plasma at that spot, not sufficient to disturb the corpuscles. As we did not see any intra-cellular forms actively liberating themselves from the corpuscle, it may possibly be that these free individuals were accidentally liberated by the rupture of the corpuscle during the process of manipulation, which would explain their apparent immobility. In the stained smears only one such free form has been found (fig. 3).

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[1] A 4 per cent. solution of osmic acid was used, to which a drop or two of acetic acid had been added.

In most of the permanent preparations made only a little searching with the oil-immersion lens is required to find a hæmogregarine. The parasite does not appear to be in quite the same period of its development in all three gurnards. In gurnards 1 and 3 the hæmogregarine is present chiefly or almost entirely in the one phase—the small type. Large forms (see below) do occur, but they are evidently very rare. Two or three individuals of the latter type have been found both in the general circulation (fishes 1 and 3) and in a smear from the kidney (fish 1), but they have not been observed in smears from the liver or spleen, and no other phases have been seen in any of the organs. In fish 2, on the other hand, in which the parasites are rather more numerous, besides the small forms many large forms occur in the general circulation, and these belong to two distinct types. Most unfortunately, as it has turned out, no smears from the organs were made in the case of this fish.

Considering first the relation of the parasites to their host-cells, we find that nearly always a single individual is present in a blood-corpuscle. Instances of a double infection occur, but they are very rare; we have only noticed two. In these cases two small forms lie side by side in one half of the corpuscle, the nucleus being displaced to the opposite end (fig. 27). The only effect which the parasites appear to have on the corpuscles is a mechanical one. In no case does the hæmogregarine attack the nucleus or cause its hypertrophy or other degenerative effect.[1] In corpuscles infected with the small forms the nucleus is generally displaced to one side (cf. figs. 1, 2, 16, 17). In the case of the large forms, the nucleus of the host-cell is often displaced quite to one side, lying at the periphery of the corpuscle (figs. 18–26). It may be slightly flattened or compressed (figs. 18–20), but shows no other sign of alteration.

In a few cases indications of the presence of a delicate membrane or envelope around the intra-cellular parasite appear to be furnished by the stained preparations in a rather interesting manner. Usually no signs of such a structure can be detected; even where, as occasionally happens, the parasite has shrunk slightly from the enclosing corpuscle, leaving a clear space around itself, it cannot be said, as a rule, that a definite sheath is apparent. But now and again the stain is deposited in a marked manner in the protoplasm of the corpuscle immediately around the parasite, which points to the existence of a layer of somewhat altered character, acting as a hindrance to the further penetration of the stain. This is especially well seen in those cases where the cytoplasm of the parasite happens to have partly or entirely shrunk (as in figs. 6, 39), leaving a deeply stained line bordering the outer edge of the clear space. We have noticed this appearance occasionally around both the small forms and the large ones of the wide type (fig. 11), but it is not shown clearly in the case of the large forms of the narrow kind, perhaps because the deeply stained cytoplasm of these individuals renders it less apparent. We regard this sheath as probably in the nature of a cytocyst, i. e. an altered layer of the blood-corpuscle.[1]

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[1] Hence this parasite does not belong to the genus Karyolysus.

In describing the structure of the parasites we may begin with the small forms. These show, on the whole, great uniformity in size and appearance. The body is nearly always oval in shape, either a fairly regular oval (figs. 39–42) or slightly pyriform, the half in which the nucleus lies being in this case somewhat narrower than the other half, and the end less broadly rounded (figs. 2, 8, 16). The average size of these small individuals—the mean being taken of several measurements—is 4·8 μ in length by 2·3 μ in greatest width on "wet" smears (sublimate-acetic mixtures, iron-hæmatoxylin), and 4·9 μ in length by 2·4 μ in width on "dry" smears (osmic, absolute alcohol, Giemsa). We regard the above figures as representing, as nearly as possible, the true or correct average size of this phase. The largest "normal" dimensions noticed are 5·2 μ by 2·6 μ, and the smallest 4·4 μ by 2·2 μ. We add "normal" because it is interesting and instructive to note one or two instances which well illustrate the considerable variation in this respect, which may be caused solely by the technique. One of them[2] is afforded by an extremely thin, "dry" smear in which both corpuscles and parasites are greatly flattened out and consequently enlarged. On this smear the apparent average size of the small form is 5·6 μ by 3 μ, and some individuals measure as much as 6·2 μ by 3·3 μ. On the other hand, in a particular "wet" smear (Schaudinn, Twort) both corpuscles and parasites are uniformly smaller than the normal average on other films made at the same time. Here the average is only 4·2 μ by 2·2 μ (cf. figs. 58, 60, 61). We are rather at a loss to account for this case, but it seems evident that shrinkage must have taken place sometime during the manipulation.

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[1] We have observed nothing so well marked as the capsule, with lines of rupture, described by Sambon and Seligmann, for instance, around certain hæmogregarines of snakes (**21**).

[2] It is, perhaps, scarcely necessary to mention that these cases are not included in the above "standard" average.

In the small type of the parasite the nucleus is relatively large. As already mentioned, it is generally situated entirely in one half of the body and near one end, but now and then it occupies a more median position (fig. 5). In Giemsa-stained preparations the nucleus appears to take up quite half the body, or even more, and to occupy its entire width or even to bulge out slightly at the sides (figs. 1, 2, 7). From preparations stained with iron-hæmatoxylin, however, it is apparent (cf. figs. 39–45) that in the former smears there is a certain amount of artificial enlargement, due to the characteristic overloading with the stain which so detracts from the value of the Romanowsky method. We reserve a detailed description of the structure of the nucleus until later, when we compare it with that of a trypanosome. The general cytoplasm appears fairly homogeneous in character, and with the exceptions to be mentioned, is usually devoid of granules. A conspicuous vacuole is frequently present; this lies about the middle of the body, generally close to the nucleus. In Giemsa smears it is sharply defined, because the cytoplasm, as a rule, is distinctly stained, either purplish or bluish in tint (figs. 1, 8, 16, 17); but in iron-hæmatoxylin preparations it is often difficult to make out, probably owing to the fact that the cytoplasm of the parasites in these smears is itself very pale and scarcely stained at all; sometimes, however, it is well seen (fig. 40). Rarely the cytoplasm contains two or three vacuole-like areas, with less sharply defined limits (cf. figs. 2, 41).

The most striking feature in the morphology of the parasites, as brought out by iron-hæmatoxylin, is afforded by one or two large granules, which take up this stain with intense avidity (cf. figs. 39, 40, 43, 46). They are often present, but not always (figs. 41, 42). When they occur these granules are by far the most prominent objects in the body, appearing always larger and much blacker than the chromatic grains of the nucleus. The granules are of about the same size whether two are present or one. They are always distinctly outside the nucleus, lying usually close to it, however, about the middle of the body. They were first noticed in the iron-hæmatoxylin preparations, and we surmised at first that they might correspond to part or all of the conspicuous vacuolar region seen in Giemsa smears, but have found since that this is not the case. In some individuals both grains and vacuole are seen to be present (figs. 1, 13, 16, 40). Sometimes one of the grains appears to lie in the vacuole, but we think that, in such a case, the grain is really outside the vacuole, lying above or below it instead of at the side (cf. especially fig. 43, where one grain is at the side, the other apparently in the vacuole). Although the grains (or single grain) usually lie close to the vacuole, this is not always so; for instance, in the parasite drawn in fig. 46 the grain is well removed from the nuclear zone, while in fig. 39 it is on the opposite side of the nucleus, near the other end of the body.

Turning next to Giemsa smears for indications of these grains, we have found that there is often considerable difficulty in recognising them with certainty. This is chiefly because none of the individuals show any signs of bodies which have taken up the Giemsa stain in the same intense manner in which the above-described granules stain with iron-hæmatoxylin. In individuals which do show granules that can be reasonably identified with those, the granules are markedly smaller in size. Hence care has to be taken not to be misled by stray, reddish-staining grains of the ordinary chromatoid character, of which occasionally one or two are present in the cytoplasm. Making all possible allowance for such, we do nevertheless find in some parasites one or two definite granules, situated close to the cytoplasmic vacuole, in a position similar to that often occupied by the granules in the iron-hæmatoxylin smears, which there can be little doubt actually correspond to those bodies. They stain dark-reddish in most smears, about the same colour as the chromatin-masses of the nucleus, whereas isolated chromatoid grains are a fainter red. Figs. 16, 17 show instances of which we feel fairly sure, as also figs. 4, 25 of a large form. In the latter the two granules contrast distinctly with the mass of chromatoid substance, of which we have more to say below. Their position in this case, some distance from the nucleus, agrees closely, it will be noticed, with that of the one grain in the iron-hæmatoxylin individual drawn in fig. 49. On the specially thin smear, already alluded to, which was only lightly stained, most of the parasites show no signs of these granules; only in one or two individuals is a round, faintly pink-staining body present, which probably represents one (figs. 8, 9).

There is another point to mention in this connection. In Giemsa-stained smears, parasites which show these granules clearly are much scarcer than in iron-hæmatoxylin smears. The explanation is probably as follows: To judge from the iron-hæmatoxylin smears, in certain of the parasites the grains (or grain) are more or less separated from the nucleus (cf. figs. 44, 46, 49), but in others they are close to it and may be in contact with it. Hence in the Giemsa films, where the nucleus is so obviously enlarged by being overloaded with stain, the granules may be swamped, as it were, and not distinguishable from the nuclear mass. We may expect, therefore, to be able to recognise them only where they are well out of the nuclear zone.

Since the granules appear distinctly smaller in Giemsa-stained preparations than they do in iron-hæmatoxylin ones, it is most likely that the former depict more nearly the true size, and that in the latter there is some overloading, due to the strong affinity the granules have for the iron-hæmatoxylin stain. This is rather important in considering the significance to be attached to these bodies. For if they were of chromatin in the ordinary sense—like the grains and masses in the nucleus—we should expect to find them apparently quite as large in the Giemsa smears as in the iron-hæmatoxylin ones; since, as is well known, the Romanowsky stain is deposited most heavily in and around chromatic structures, with resulting enlargement of size (cf., for instance, the kinetonucleus of a trypanosome). The fact that this is obviously not the case here suggests that the granules are not chromatic structures.

In this connection certain of our Twort preparations are very instructive. As has been shown by Minchin (l.c.) the Twort stain is in one respect superior even to iron-hæmatoxylin, in that chromatic elements can be distinguished from others by the fact that they alone stain red, everything else being green. These particular Twort smears were examined soon after being made to see if they showed granules corresponding to the ones we had already found in iron-hæmatoxylin films. In several individuals one or two granules were seen, which we regarded as those for which we sought. They were small, however, and not particularly conspicuous. They were very faintly stained red, nothing like so deeply or sharply as the grains in the nucleus. Some of the individuals showing these granules were noted and sketched at the time, and then the smears were put aside to work out on our return to England. On finding the same parasites again recently, in order to draw them, we could no longer see the red granules in any of the individuals marked. All signs of them have vanished, although the red of the nucleus has not faded at all. In one or two cases, however, in about the position which was occupied by the red granules (according to our sketches), small rounded areas, somewhat diffuse in outline, can be made out, staining a rather deeper green than the surrounding cytoplasm (cf. fig. 59). It may be that these greener areas mark the position of the structures which stain so intensely with iron-hæmatoxylin. In the great majority of the individuals stained with Twort, however, the body is uniformly pale green in colour, and cannot be said to show any indications of the granules.

To sum up, we regard the above-described characteristic bodies as composed, at any rate, chiefly of achromatic material. Our opinion is based on the one hand upon a comparison of their staining reactions to iron-hæmatoxylin and to Giemsa, and on the other hand upon the fact that they show very little, if any, real affinity for the neutral red of Twort's stain. It may be pointed out that, besides the fact of their being frequently double, these grains differ from kinetonuclei in their staining reactions. After Giemsa, they appear only reddish and have not the characteristic dark purple or almost black colour associated with true kinetonuclear elements; compare, for instance, the intensely staining chromatic grain described by Woodcock (**23**) in the case of a Halteridium of the chaffinch, which is quite distinct from the ordinary nucleus. Again, their appearance after Twort's stain shows no resemblance at all to that of kinetonuclei. With regard to the staining of these grains by Twort and iron-hæmatoxylin, one of us (E. A. M.) has found an interesting parallel in the case of the blepharoplast (basal granule) of the flagellum of Trypanosoma lewisi. In the multiplying (not the adult) forms of this parasite the blepharoplast appears after Twort's stain as a diffuse green patch, but after iron-hæmatoxylin it is seen as a definite black granule. This comparison suggests that the grains seen in H. rovignensis are centrosomic in nature, but apart from the above facts we have no further evidence to bring forward in support of this conclusion.[1]

We pass now to the consideration of the large forms of the parasite. These are, as already mentioned, of two distinct types: (1) Long, comparatively slender individuals, often slightly curved or bow-shaped, which possess a small nucleus (figs. 18–21); and (2) broad forms, oval or bean-shaped, which have a much larger nucleus (figs. 22–26). In the wide forms both ends of the hæmogregarine are generally similar and more or less bluntly rounded. In the slender forms, on the other hand, one half of the body is usually narrower than the other and tapers towards its extremity, the opposite end being bluntly rounded. The more pointed end may be curved or partially bent up on itself (figs. 18, 21), this being most likely due to limitations of space; the drawn-out corpuscle is apparently incapable of being stretched to the full extent of the length of these forms. (We have not seen any phases in this parasite, it should be said, indicating the development of a definite U-form such as occurs in many hæmogregarines.) The average size of the slender type is $12\,\mu$ by $2{\cdot}1\,\mu$; and individuals up to $14\,\mu$ long have been observed, the breadth in this case being $2{\cdot}2\,\mu$. The stout forms average $10{\cdot}6\,\mu$ by $3{\cdot}4\,\mu$, and their extreme size is about $11\,\mu$ by $3{\cdot}5\,\mu$. The size of the nucleus differs greatly in the two cases, and this is a constant feature. In Giemsa smears the nucleus of the slender type averages $2{\cdot}6\,\mu$ by $1{\cdot}7\,\mu$ and that of the wide type $4{\cdot}8\,\mu$ by $2{\cdot}9\,\mu$; in iron-hæmatoxylin preparations the former is $2{\cdot}5\,\mu$ by $1{\cdot}4\,\mu$ and the latter $4\,\mu$ by $2{\cdot}2\,\mu$. In the slender forms the nucleus is always in the narrower, tapering half.

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[1] We have noticed one or two references to the occurrence of bodies in other hæmogregarines, which may perhaps relate to a similar organella. Thus Miss Robertson (**18**) describes and figures two large oval "red bodies," staining red with Giemsa, in H. vittatæ; and again (**19**) she mentions the occurrence of an eosinophile body or vacuole, beside which is a sharply staining grain, in a hæmogregarine from Pleuronectids. Whether, on the other hand, the "Plastinkerne" described and figured by Prowazek (**16**) in H. platydactyli also represent a corresponding body appears more doubtful.

The structure of the nucleus appears practically the same in both the stout and slender individuals; it agrees closely also with the structure of the nucleus in the small forms. The cytoplasm of these large forms, however, differs greatly in character from that of the small parasites to judge from the effect produced by the Giemsa stain. The difference is especially noticeable in the case of the slender forms. Here the broader half of the body (not that in which the nucleus is situated) is nearly always more or less completely filled with some substance which stains red, and which may indeed appear at times almost as deeply and intensely stained as the nucleus (cf. figs. 19, 21). Often the colour increases in depth regularly towards the broad end, as if the substance which attracts the stain were most concentrated in that region of the body (fig. 18). From the effect produced by the Giemsa stain it would seem as if the mass were of a finely granular character and consisted of chromatoid material. Some of the wide individuals show little or no indications of this substance (figs. 22, 23), but in others the condition is present to a greater or less degree (figs. 24, 25). It is never so prominent as it frequently is in the slender type of form. It is somewhat remarkable that neither iron-hæmatoxylin nor Twort show anything at all corresponding to this appearance so far as can be seen. After Twort's stain the body of the large form either appears finely granular and faintly tinted green (fig. 62) or else it is very pale, scarcely, if at all, stained (fig. 61).[1] Yet it is quite evident that there must be something more than merely the ordinary cytoplasm present to account for the appearance seen after Giemsa.

We are inclined to doubt whether the characteristic granules above described occur so frequently in these large forms as they do in the small ones. They are not visible, for instance, in either fig. 50, of a slender individual, or in fig. 48 of a broad one. One granule is seen in the stout form of fig. 49, however, and a couple in that drawn in fig. 25. Also in the slender parasite in fig. 4 there are a couple, but this is the only full-sized individual of this type in which we have made them out with certainty. Fig. 45 represents a slender form of intermediate size, and this shows a single prominent granule.

Nearly all the individuals of the slender and broad types appear to be full grown. We have been able to find, however, two or three examples of what are undoubtedly young, growing individuals of these forms. They occur in slides of the series containing many of the large forms. Two young parasites of the slender type are seen in figs. 12, 13; and a young individual which would probably have developed into a stout form is drawn in fig. 14. One of the slender forms shows very conspicuously the two characteristic granules; and in the small broad individual we think it not at all unlikely that one or both of the grains at the edge of the nucleus, on its inner side, may represent the same bodies. Individuals which are somewhat larger, but still distinctly intermediate in size, are seen in figs. 45 and 15, the former being of the slender type, the latter of the broad type.

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[1] The shading, often irregular, of the iron-hæmatoxylin parasites in figs. 45 and 49, for instance, is meant to indicate the slightly varying thickness of the densely stained cytoplasm of the corpuscle lying over the parasite.

We have not found any other phases of the hæmogregarine, besides those above described, in our preparations. In the gurnards from which smears of the internal organs were made the parasites happened to be nearly all small forms, and large forms are very scarce. In these cases, although preparations from the liver, kidney and spleen have been searched, no signs of schizogony have been noticed.[1] Perhaps if smears had been taken from these organs in the case of the gurnard in which the large types of form are frequent, multiplication phases of a particular kind might have been found.

It is important to note that we have never found any individuals of either of the large types free from the blood-corpuscle. Further, in only a solitary instance has one of the small forms been noticed free (fig. 3). This occurs on a smear from the kidney. The parasite is not very vermicule-like, and resembles the small intra-cellular type of form. This scarcity of free forms quite agrees with our observations of the parasites in the living condition (cf. above, p. 115).

General Considerations.

We have now to consider what is the significance of the different phases observed, in what relation do they stand to one another, and how do they compare with the known forms of other hæmogregarines ?

The ordinary small forms doubtless represent an early stage of the infection, and may be regarded as derived from sporozoites which have penetrated the blood-corpuscle. They are for the most part very uniform in size and appearance and probably destined to become schizonts.

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[1] The only possible indication of commencing schizogony which we have noticed is found in two or three of the small forms in a preparation from the infected gurnard examined. The nucleus of the parasite shows a constriction about the middle, which causes it to appear somewhat dumbbell-shaped (fig. 28). This may, perhaps, signify commencing nuclear division, prior to fission of the parasite, but we do not feel at all certain upon the point.

The large forms are, we consider, of two distinct types, and not directly connected with one another—that is to say, an individual of one kind, e.g. a broad form of parasite, does not pass into one of the other kind, the slender type, by a process of elongation and simultaneous bending-up, such as is described by Börner (**4**) in H. stepanowi; nor, on the other hand, is the broad form to be derived from the slender type by a process of change comparable to that frequently described among Reptilian hæmogregarines, where a U-shaped form gives rise, by the fusion of its two arms, to an oval or bean-shaped form (cf., for example, H. tunisensis of Bufo mauritanicus, Billet [**2**], H. bagensis of Emys leprosa, Billet [**3**], etc.). Our reasons for regarding the two large forms of H. rovignensis as independent are three: In the first place, we have noticed no transitional forms indicating such a connection as just mentioned; secondly, there is always a well-marked difference between the nucleus of forms belonging to these two types; and lastly, young individuals of each type are clearly distinct. We suggest that these large forms show sexual differentiation, the slender type with the small nucleus being of male character, and the stout form with the large nucleus being of female sex.

With regard to their origin, it is very probable that they have been developed from two forms of merozoites, smaller and larger, for the young individuals of the slender or male type in figs. 12, 13 are manifestly different from the other small forms, and not likely to have arisen from them. There is one point, however, in this connection which at first was not at all clear. In the second gurnard examined (figs. 12–14, 16–26, 44–50) the number of the young parasites found belonging to the type which we regard as male is very small indeed as compared with the number of the ordinary small forms present, whereas about equal numbers of the full-grown parasites of both types occur, and these are fairly plentiful. We think the most probable explanation is that we have to deal here with more than one infection. The great majority of the small forms must be regarded as the early stages of a new or recent infection; the forms showing sexual differentiation, on the other hand, belong, we think, to an older infection. Most of them occur as adult large forms, and only a few are to be met with as young or intermediate-sized individuals. The young parasites of the female type (belonging to the older infection) are probably not very different in appearance from the numerous small forms of ordinary type (of the recent infection), and thus are only distinguishable where they are beginning to grow into the stout type (fig. 14). The two forms of merozoites, which give rise in time to the large individuals, have doubtless been developed by a schizogonous process,[1] probably occurring in the internal organs.

The large forms themselves must represent one of two phases, schizonts or gametocytes respectively. If, according to the first of these interpretations, we have here micro- and macro-schizonts which will give rise again to a fresh series of micro- and macro-merozoites, it is evident that sexual dimorphism is apparent throughout the schizogonous series of generations (as is known to occur among certain Coccidia, e.g. Adelea, Cyclospora). If, on the other hand, our two types are micro- and macro-gametocytes respectively, we have no indications with regard to the nature of the ordinary schizogony (or fission) in this parasite, which may be very likely all of one kind, that is to say, "indifferent" in character, with no sexual dimorphism manifest (as in many Coccidia and all known Hæmosporidia). In this case it will only be in the last generation produced by schizogony that sexual dimorphism appears, in the formation of what are really the young micro- and macro-gametocytes (cf. above).

Unfortunately, from our own preparations alone, we cannot pronounce definitely between these two views. When, however, we compare the facts which we have learnt concerning H. rovignensis with what is known in the case of other piscine hæmogregarines, we are strongly inclined to consider the latter view the correct one, and that the two distinct types of form are micro- and macro-gametocytes. Fission or schizogony is now known to occur in many piscine hæmogregarines, and in most of the instances described it appears to be of one kind, no indications of sexual dimorphism being mentioned (cf. H. bigemina, Laveran & Mesnil [**6**], H. quadrigemina, Brumpt & Leb. [**9**, fig. 3, p. 382], H. simondi, Lav. & Mesn. [**6**], etc.). Neumann, in his account of piscine hæmogregarines (**15**), regards the schizogony in these instances as resulting in the formation of "gametes," but does not attempt to explain why only one kind is described and figured. Further, in his account of H. polypartita from Gobius pagenellus, he regards similar crescentic forms, four of which are developed in a blood-corpuscle, also as "gametes." He endeavours to show that these forms exhibit sexual differentiation, but such distinctions as are apparent in his figures seem to us to be due merely to slight differences in size (or age) and in tint of colour (otherwise degree of staining). We do not find anything approaching the pronounced and constant differences, both in form and in the size of the nucleus, which are shown by the large types of H. rovignensis. We certainly consider the curved forms of H. polypartita—equally with those of Neumann's other new species, H. clavata—as "merozoites," probably really "indifferent" in character,[1] and quite comparable with those described in the above-mentioned parasites.

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[1] The process is, perhaps, comparable to the formation of merozoites of two sizes in cysts of Karyolysus (cf. Labbé [5]), both as regards the development and the significance of the small elements formed.

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[1] It is quite possible, of course, that in some hæmogregarines the schizogonic forms exhibit sexual differentiation. Up to the present, however, we do not consider this has been shown to be the case. Wenyon (**22**) has figured "barillets" both of micro- and mega-merozoites in connection with H. gracilis from the liver of Mabuia. Certain of Wenyon's figures suggest strongly the schizogony of a Coccidian, and we think this explanation is not at all unlikely, both in

There is another point which is of considerable importance. In all the above instances these long, slightly curved forms (adult merozoites) were readily observed free, as "vermicules," in smears as well as in the living condition. This is quite natural if, as we consider, these vermicules are ready to penetrate a fresh host-cell, probably in an internal organ, and there give rise by schizogony to the sexually differentiated forms. We think it will be useful to distinguish these free individuals as schizokinetes, meaning thereby a temporarily motile schizont.

In striking contrast to the above cases we have the entire absence, so far as we are aware, of free individuals of either of the large types of H. rovignensis, although the great majority of them seem to be full-grown and mature. We have no grounds whatever for thinking that these individuals become free from the corpuscle while still in the fish.

This difference in behaviour also points to these large forms of the Trigla-parasite being gametocytes and not schizokinetes. On the supposition, which is most probable, that the sexual process of these hæmogregarines takes place in a leech, these is no reason why we should expect to find gametocytes liberated in the blood of the fish, because they are awaiting the stimulus of the invertebrate host before being set free from the corpuscles. We may compare in this respect other intra-cellular blood-parasites, for instance, Halteridium, Proteosoma, or Leucocytozoon of birds, with which we have had much to do. If a drop of blood containing these parasites is taken and smeared quickly, scarcely any of the ripe gametocytes present have ruptured the host-cell and become rounded off. It is only when the blood is allowed to cool for a short time in the living condition that the sexual forms become free, as indeed is well known. It may be said that if the large forms of H. rovignensis are gametocytes, we ought to have seen some of them at any rate become free in the living preparations examined. This does not follow at

view of the situation and also on account of the nuclear structure (cf. also below, p. 149, footnote).

all, for the particular stimulus which effects the liberation of these elements in the above cases is here lacking, namely, the fall in temperature. Lastly, it is not out of place, perhaps, to refer in this connection to Miller's account of "Hepatozoon" in rats (**10**). In this case the sexual forms (which apparently show little or no differentiation) are encysted in lymphocytes. Miller found that when blood containing the parasites was mixed with the expressed juices of the mite (Lelaps), it was ten to thirty minutes before the host-cells were dissolved and thirty minutes or more in addition before the gametocytes were liberated from their capsules and became motile vermicules.

From all these facts we conclude that where large free vermicules of a hæmogregarine are found in the circulating blood, at least in fishes, they are schizokinetes, which have yet to give rise to the true sexual forms, and not themselves the gametocytes ("gametes" according to Neumann). It is very doubtful whether the full-grown gametocytes of hæmogregarines ever become free until the blood is drawn from the body.[1]

Characteristics of H. rovignensis nobis.—The principal characters of this species from Trigla lineata, so far as we have been able to ascertain them, are as follows: A very small parasite, one of the smallest piscine hæmogregarines yet described. Schizonts usually oval in form. Average size (full grown?), before fission has commenced, 4·8 μ by 2·5 μ. Gametocytes large and well differentiated. Female forms wide, ovoid or bean-shaped; average size 10·6 μ by 3·4 μ. They possess a large nucleus. Male forms fairly slender, with one end somewhat club-shaped, the other end usually slightly recurved; average size 12 μ by 2·1 μ. These possess a small nucleus. Individuals of all types may show one or two characteristic granules, extra-nuclear in position, and most probably achromatic in nature; they are particularly prominent in parasites stained with iron-hæmatoxylin, which stain they take up with great avidity.

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[1] From the published descriptions and figures of reptilian hæmogregarines which we have seen we consider it most probable that a similar state of affairs obtains in their case also.

II. Trypanosoma sp. (cf. triglæ) in Trigla lineata.

(Figs. 29–31, 51, and 65.)

In the three gurnards which were infected with Hæmogregarina rovignensis a trypanosome was also found. This parasite was always very rare, and never more than two individuals could be seen in a cover-slip preparation of living blood. Neumann (**15**) gives only a very brief notice of T. triglæ, from a single Trigla corax, and does not remark upon the strength of the infection in this particular case, but he states at the beginning of his paper that as a rule trypanosomes appear to be very rare in the blood of infected fish.

Observed in the living condition, the trypanosomes performed very active movements of contortion, but did not actually displace themselves much in the blood-fluid. The movements were of the wriggling and twisting kind so characteristic of fish trypanosomes. The body would be coiled into a spiral or **S**-shape and then unbent again only to become twisted in the reverse sense with the greatest rapidity. Occasionally the parasite would burrow into a mass of corpuscles and pass through them. Particular parasites, whose position was noted, were found to remain alive from five to eight hours in the drawn blood or cover-slip preparations. Their movements had become extremely sluggish by the end of this time, but no alteration in form was noticed. Only in a single case was a parasite seen alive after a longer interval (twenty-four hours); the body of the trypanosome was then bent up and motionless but the flagellum waved feebly, showing that the parasite was still alive. The parasites did not live any better in preparations in which the blood had been mixed with a drop of salt-citrate solution or of sea-water. In remaining alive such a short time in drawn blood this trypanosome differs markedly from T. raiæ, as will be seen from our statements below.

Owing to the scarcity of this parasite very few individuals are present on our stained preparations. At the most two occur on a film; more usually only one has been found, and on some smears there appear to be none. All the parasites seen belong to one type and show no pronounced variation in size. Individuals on "wet" smears are generally a trifle smaller than those on "dry" ones; we think the former are slightly contracted. The average size of the trypanosome, as seen on Giemsa-stained smears, is 59 μ in total length (i.e. inclusive of the flagellum) by 4·5 μ in total width (inclusive of the membrane). The free flagellum averages 8 μ in length. Fig. 29 shows a typical example of the parasite, with dimensions almost as given. The longest parasite observed (fig. 31) has a length of 62 μ and a breadth of 4·7 μ. The length may be really 2 or 3 μ longer, as the flagellum is very faintly stained, and its free portion probably continues a little farther than can be made out. On the other hand the parasite from an iron-hæmatoxylin film drawn in fig. 51 has a total length of only 54 μ and a width of only 4 μ, inclusive of the membrane; but here also the free part of the flagellum is so faintly stained that we cannot be certain its entire length is represented.

All the trypanosomes on our slides belong to this long, slender type. The flagellar extremity is narrow and finely tapering (figs. 29–31), a feature which is more usual in these parasites of marine fishes than in those of fresh-water ones. The distance of the kinetonucleus from this end of the body is generally about 6 μ, and may be as much as 7 μ. The free part of the flagellum at the opposite end is comparatively short, and varies from 6 to 10 μ. The trophonucleus is generally in the flagellar half of the body (figs. 29–31), but may be occasionally more centrally placed (fig. 30). The undulating membrane is well developed. It appears under two aspects. In the first, which we think represents the more natural condition, it shows well-marked folds and pleats, of which there are six to eight, of varying prominence and depth (figs. 29, 31). In other cases the membrane appears as an extensive flap or fin, with a slightly wavy border, and widest about the middle of the body (fig. 30). We think the difference between these two aspects is more apparent than real, due to a large extent to the flattening out of the parasite on the slide, in the second case. On the other hand, in the few individuals we have observed on " wet " films the membrane appears narrow and inconspicuous (cf. figs. 51, 65), and we should certainly be inclined to say it had undergone some shrinkage here.

One or two remarks which we have to make upon the nuclear structure may be deferred until we consider the trypanosome-nucleus generally in the second part of this paper. The only detail with regard to the general cytoplasm that requires mention is the occurrence in certain cases of numerous granules. These granules are only noticeable in parasites on Giemsa-stained smears. In some individuals (fig. 31) the cytoplasm is quite free from them; in others there are a certain number of small dark granules, chiefly in that part of the body lying between the two nuclei. The flattened-out individuals, however, are rendered conspicuous by the presence of numbers of large granules, apparently occupying most of the body (fig. 30). Moreover, many are seen lying apparently in the membrane. These granules stain a purple colour of a somewhat deeper tint than the lilac of the cytoplasm.

The trypanosome from Trigla corax, to which Neumann has given (l. c.) the specific name of triglæ, is described very briefly and without any figures. According to Neumann's account the parasite is of a different type of form from that which we have above described. Its total length is about the same, namely, 60 μ, but the free flagellum is rather longer, being 15 μ. The chief difference, however, is in the breadth, which appears to be much greater. Neumann gives the width of the body as 8 μ and that of the membrane as 4 μ, the entire width being thus 12 μ as compared with an average of 4·5 μ in the case of our form.[1] Further, the aflagellar end of the body is short and somewhat blunt, as indeed is often the case in these "stumpy" types of trypanosome, and the membrane does not show well-developed folds.

At first sight these two trypanosomes, from different species of Trigla, might be considered to belong without doubt to different species; and probably many authors would not hesitate to give both parasites a distinct name. In our opinion this would be decidedly premature, for we think it quite likely that both are merely different forms of one and the same species. Polymorphism is now known to be of common occurrence among trypanosomes. To give only one or two instances, Minchin (**11**) has recently shown clearly, by photographs, the marked (sexual) polymorphism in T. gambiense, while one of us (H. M. W.) has been struck by the polymorphism, of a character quite similar to that implied in the case before us, which occurs in an Avian trypanosome. We do not intend here to ascribe any special sexual significance to the "stout" (wide) form of the Trigla trypanosome; we suggest, however, that Neumann's form is a particular type of the parasite we have described above, the latter being the one which we are inclined to regard as the more "ordinary" type. Hence our reasons for including the trypanosome from Trigla in the species T. triglæ.

In connection with this point, it may be remarked, it is especially among piscine trypanosomes, where the parasites attain to such large dimensions that marked variations in form and appearance may be expected to occur, due either to young forms or to different types of the parasite. It seems to us that there has been too much tendency to ascribe hard and fast limits to the size of a specific trypanosome. Many authors, in describing new species, appear to have overlooked the possibility of considerable variation in size and appearance, and as a result have given fresh names to parasites which are in all probability only phases of trypanosomes already known from the same or closely allied hosts. We have little doubt that Neumann has made such a mistake in distinguishing his "Trypanosoma variabile" from T. raiæ of skates.

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[1] It is quite possible that the real width is not so much as these figures indicate; for we have found that it is especially in such stout or stumpy types that flattening-out in dry smears may be most appreciable and most liable to give an incorrect idea of the true width of the parasite.

III. T. RAIÆ FROM RAIA SP.

(Figs. 32–38, 52–57, and 66–68.)

As this parasite has been described already by several workers, we need not give a general account of it here. One or two points of interest have been observed, however, which may be mentioned. In the fish examined the trypanosomes were exceedingly abundant, but no dividing forms were seen, nor were any hæmogregarines found.

With regard to the behaviour of the trypanosomes in freshly taken blood under a cover-slip, we found that they remained alive and active for a much longer period than did T. triglæ kept in a quite similar condition. Many were seen quite unaltered, but undergoing less active moments, after twenty-four hours. Also after fifty-four hours several were seen, their movements being now sluggish. At the end of seventy-two hours only four were found, two of them being individuals which had been noted and marked after twenty-four hours. They showed no alteration, but their movements were very slow and feeble, being confined to little jerks of the flagellum and a very slight twisting of the body. Three of these individuals were seen again on the fourth day, after ninety-six hours, when they appeared in much the same condition. Lastly, on the fifth day one trypanosome only was still seen living, extremely feeble and moving very slightly at intervals. The remarkable point is that none of these parasites showed any alteration in form; nor during the earlier periods, when several individuals were still alive and fairly active, did we notice anything comparable to the rounded-off phases described by Miss Robertson (**17**). It is probable that the percentage of individuals which undergo this alteration on the slide is very small compared with the number that do so when the parasites pass into the leech.

The trypanosomes in our permanent preparations show considerable differences in size. On Giemsa-stained smears the largest individual observed has a total length of 72 μ and a breadth of 5·6 μ, including the membrane. The corresponding dimensions of the smallest form seen are only 55 μ by 4 μ (fig. 33). These two extremes are connected by intermediate forms of varying size (c f. figs. 34, 35). The average size works out at about 67 μ by 5·2 μ. The free flagellum varies from about 10 μ to 15 μ, with an average length of 13 μ. The length of the flagellum does not seem to stand in any very close relation to the size of the parasite, and now and again is shorter in a large individual than in one of intermediate size. The largest trypanosome noticed on "wet" films stained with iron-hæmatoxylin is drawn in fig. 53; it is a comparatively wide, plump individual, which would probably have seemed even wider on a Giemsa-stained smear. It measures 65 μ by 6·6 μ. It is probably somewhat longer in reality, for the free flagellum, which is unusually short in the drawing, comes into contact, at the point where it apparently ends, with a corpuscle which is stained deep black; although it probably runs across this for some distance, its course cannot be followed. Other parasites on iron-hæmatoxylin-stained films are seen in figs. 52–56. They are mostly a little shorter than the parasites on "dry" films; this difference is most noticeable in comparing the body-protoplasm, for the free flagellum itself is in most cases actually longer and averages 14·8 μ against 13 μ on the dry smears. We are inclined to think this is due to the contraction of the general cytoplasm to a greater extent than the entire flagellum (i. e. the flagellar border + free flagellum) in iron-hæmatoxylin films.

There is another rather interesting point brought out by a comparison of the trypanosomes fixed and stained by the two methods, which, incidentally, may also help to explain this difference in apparent length. The great majority of the individuals on the "wet," iron-hæmatoxylin-stained films are in a different position from those on the "dry," Giemsa-stained smears. In the former they are usually found in a twisted S or corkscrew-like position (figs. 52–56), while in the latter the parasites are nearly always simply rolled or coiled up to a greater or less extent (figs. 32–37). Now, in life the trypanosomes are generally observed in a twisted or S-shaped condition, and only rarely, and as it were transiently, in the form of a simple C. We may conclude, also, that death and fixation are at least as instantaneous in the case where the parasites actually come into contact with sublimate and acetic as in the case where the slide is placed in a tube containing osmic vapour. Hence we consider that the position of the parasites on the wet films approximates most nearly, as a rule, to that in which they were the instant before death. What is the cause, then, of the parasites assuming the very different rolled or coiled-up form on "dry" films?

The manner of attachment of the undulating membrane to the body has an important bearing upon this question. Figs. 52, 53, and 56, from iron-hæmatoxylin slides, show very clearly that in these individuals the undulating membrane was wound spirally round the body at the instant of death. The flagellar border runs now under, now over the general cytoplasm, and in fig. 56 it is seen to run twice under. Are we to regard the membrane as actually attached spirally to the body (when the latter is in a "passive" condition), or as being merely twisted round it at the time by the voluntary contortion of the protoplasm? We think the latter view affords the true explanation. The appearance of the parasites on Giemsa-stained films gives probably a fairly correct representation, from a morphological point of view, of the manner of attachment of the membrane—that is to say, it lies along one side of the body, more or less in one plane. The membrane itself, especially on its outer side, is longer than that part of the body to which it is attached. In life the membrane is usually twisted in a spiral fashion round the body by the voluntary contortion of the protoplasm, this being in all probability effected by the contraction of myonemes. Minchin (**13**) has recently published figures clearly showing myonemes in T. percæ and T. granulosum, and we have no doubt they are present in other fish-trypanosomes, though we have not had the good fortune to see them in T. raiæ. In wet films the parasites have retained their twisted position. In "dry" smears, on the other hand, the body becomes untwisted, and, at the same time, passively or mechanically coiled up in one plane, by the mere fact of the attached membrane being longer than the body is.

We do not think this different behaviour on "dry" smears is to be explained by a flattening-out process due to actual drying. In the first place, in our procedure, the slides are removed from the osmic-acid tube and placed in absolute alcohol before the moisture dries off from the greater part, at all events, of the slide; it is only along the edges that drying sometimes occurs. And after the smear has been hardened in alcohol little or no alteration, we consider, takes place in the form of the parasites, even though the smear is allowed to dry off ultimately. In fact, as Minchin has already shown in his account of the technique in connection with T. lewisi (**14**), this method is probably the best for the general form and size of the parasites. Secondly, now and again where the body of the parasite really appears to be somewhat flattened out due to an actual drying at first, this **C**-form is not shown (cf. fig. 30 of T. triglæ). Indeed, this process of untwisting and coiling would seem to require the presence of a film of moisture for its accomplishment. The following explanation appears to us the most probable. In fixation by the "wet" method, both death and fixation are practically instantaneous. In fixation by osmic vapour, on the other hand, death probably occurs appreciably before fixation. In the twisted condition, during life, the flagellar border of the membrane is probably to a certain extent in a state of tension, from which it relaxes, in virtue of its elasticity, on the death of the body; in so doing it automatically unwinds the body, at the same time causing it to become more or less **C**-like, before actual fixation occurs. In this connection it should be pointed out that Danilewsky (4A), who studied trypanosomes carefully in the living condition, frequently figured them with the undulating membrane spirally wound round the body, but in some cases he shows it attached along one side of the body.

Lastly, if, as we have been led to consider, the parasites on wet films are generally in a spirally twisted condition, we might expect to find a slight shortening in length; this, together with a certain amount of contraction due to shrinkage, would be sufficient to explain the difference in average length between the parasites on wet films and those on dry ones.

In many of the parasites on Giemsa-stained smears numbers of small bodies occur, which appear to be prominent granules (fig. 37). They are deep black at the middle focus, but are bright and glistening at the upper focus. They are not comparable to ordinary chromatoid granules, which stain more or less red in colour. Moreover they are most abundant in the aflagellar part of the body, especially between that extremity and the kinetonucleus, a region which is generally free from chromatoid grains. They are also scattered throughout the body, and some, which cannot be distinguished by appearance from the others, lie occasionally in or on the undulating membrane. In wet films, stained either with iron-hæmatoxylin or with Twort, the same bodies, if present, are not nearly so conspicuous. In the body generally no sign of them is to be seen; but near the aflagellar end, which is often slightly vacuolated in character, a certain number of granules can be seen, not stained very differently from the cytoplasm (figs. 54–56). We are not sure, however, if these granules are the same.

Returning to the parasites on dry smears, we have recently noticed the peculiar fact that, since the individual of fig. 37 was drawn, all the black granules have vanished, leaving only small, clear areas, like spaces, in the position in which they were. In fig. 34 is another parasite which showed originally a very similar condition as regards the granules; this has been drawn since they disappeared. Two or three of the granules are still seen, and the small vacuoles indicate the position originally occupied by many others. It seems most probable that these black "granules" are really minute globules of oily or fatty substance, which are blackened by the osmic acid used in fixation, and are liable to be dissolved away by the frequent washings with xylol given to the slide, of course after immersion-oil has been upon it.

We entertain no doubt that this trypanosome belongs to the species T. raiæ, Lav. and Mesn. These authors, in describing this species, gave its size as from 75 μ to 80 μ in total length, by about 6 μ in width (inclusive of the membrane). Apparently, as has been so often the case, the species was characterised solely from the full-grown individuals of the ordinary type which were encountered, and no reference is made to young forms or to variations in type. Further, Laveran and Mesnil found trypanosomes which they regarded as belonging to the same species in four species of Raia, namely R. punctata, R. mosaica, R. clavata, and R. macrorhynchus. We do not know the name of the species of Raia in which we found the parasite. The dimensions of the largest individuals we have observed, however, are only very slightly less than those above mentioned, and the general appearance of the parasites, as shown in our figures, agrees so closely with that of the individual figured in Laveran and Mesnil's original account (7) that there is every probability that the trypansome is the same in the two cases. Neumann (**15**) has given the name T. variabile to a trypanosome from R. punctata, principally or solely because he has found variations in size and form in the parasite which are not referred to by Laveran and Mesnil; though he states more than once that his parasite resembles T. raiæ closely and in its largest form agrees with that trypanosome. As we have described above, we have found forms of T. raiæ very much smaller than the full-sized ones; and many of Neumann's figures of T. variabile resemble strongly these smaller forms. In fact, neither from Neumann's description nor from his figures is there any reason to suppose that T. variabile is not a synonym of T. raiæ.

COMPARISON OF THE NUCLEAR STRUCTURE OF A HÆMOGREGARINE WITH THAT OF A TRYPANOSOME.

A most interesting and important result of our study on the above-mentioned blood-parasites of fishes is afforded by the evidence it has given us of the essential difference between the nucleus of a hæmogregarine and the trophonucleus of a trypanosome. This difference is brought out forcibly by all the three methods of technique employed, though, of course, one method may show a certain feature or detail better than another.

Considering first the case of Hæmogregarina rovignensis in Trigla sp., the conclusion arrived at by comparing and combining the impressions given by different stains is that the nucleus in this parasite consists of a regular or irregular meshwork or reticulum, itself chromatic or impregnated with chromatin, on which are suspended chromatin grains and masses of varying size and form. The reticular ground-work is best seen in iron-hæmatoxylin or Twort preparations (figs. 39–50, 59–64); in the latter it is always distinctly red (chromatic) in colour. The limit or border of the nucleus appears to be itself part and parcel of the reticulum, the peripheral segments of the latter being usually arranged so as to give a fairly regular oval contour or "membrane." This structure is well shown in figs. 48 and 49 of large forms. Hence one cannot speak here of a true nuclear membrane as something distinct and separate from the general nuclear substance. This "membrane" also has numerous chromatic granules strung upon it; these are generally smaller than those occurring in the more central parts of the reticulum. The chief chromatic aggregations sometimes tend to run together, or to lie in short streaks. In no case have we observed any signs whatever of a definite central body or karyosome in the nucleus.

In individuals stained very faintly and sharply on the particular Giemsa-stained smear to which allusion has been made several times, the above-described characters of the nucleus can be made out quite well (cf. figs. 5–10). In such cases the picture represents fairly accurately the true condition. In other individuals, however, slightly more deeply stained, the nucleus appears more granular and already somewhat "blotchy"; this is due to the enlargement of the chromatic grains and to the deposition of the red stain in the nuclear sap, more or less occluding and obliterating the reticulum. This leads on naturally to the appearance generally seen in deeply stained Giemsa smears of a dense mass, staining red or purple, in which bodies and streaks still more darkly coloured can be made out, representing the chromatic grains.

We may add that we have been struck by the considerable resemblance between the nucleus of the parasite and that of its host-cell; this will, indeed, be apparent from many of the figures (fig. 14).

Turning now to the trophonucleus of a trypanosome, we find a remarkable contrast. T. raiæ being a very large trypanosome and possessing a correspondingly large nucleus is a most advantageous form to study for this purpose, since the various nuclear details—particularly of the karyosome—can be made out more readily than in the case of a comparatively small parasite, such as T. lewisi, for example.

Our description is based upon the appearances yielded after iron-hæmatoxylin and Twort, for in this case—far more so than when considering the hæmogregarine nucleus—it would be difficult, if not impossible, to arrive at what we regard as the correct interpretation of the nuclear structure by studying the Giemsa appearance alone. Having obtained a fairly accurate idea of the nuclear constitution from iron-hæmatoxylin and Twort preparations, we can then interpret the widely different picture seen after Giemsa. Miss Robertson

(17) has recently described the nuclear structure in certain developmental forms of a trypanosome occurring in Pontobdella, which trypanosome she regards (and we think correctly) as T. raiæ. Our own observations quite agree with her account; we are able, perhaps, to add a few more details with regard to the karyosomatic mass. (Our main purpose, as we have already said, is to emphasise the contrast between this type of nucleus and that of a hæmogregarine.)

The nucleus is very generally oval in shape and always possesses a well-defined, regular contour. Its size varies not inconsiderably (cf. figs. 52–56), and, as might be expected, is in accordance with the size of the parasite, small- or intermediate-sized individuals having a smaller nucleus than the large ones. The size may be as small as $2{\cdot}4\,\mu$ by $1{\cdot}7\,\mu$, or as large as $3{\cdot}2\,\mu$ by $2{\cdot}6\,\mu$. The greater part of the nucleus is occupied by a prominent deep-staining body—the karyosomatic mass. Around this appears a practically clear space, which is bordered or limited by a sharply marked line, the nuclear membrane. Any space or halo surrounding this on the outside again, as sometimes occurs (cf. figs. 52 and 54), is most likely a shrinkage-space. Delicate rays, sometimes four or five in number, sometimes more, proceed from the central mass to the membrane; these are usually very faintly stained, but can be made out with a good illumination, especially in iron-hæmatoxylin preparations. Both membrane and rays are always green after Twort (figs. 66, 67, 68*a*). Hence we may regard them as achromatic in structure. The rays are probably comparable to a linin framework for the support of the karyosome. The membrane is a much more definite structure than in the case of the hæmogregarine-nucleus. In this parasite both membrane and rays appear to be, as a whole, remarkably free from chromatin, very different in this respect from the chromatic reticulum of the hæmogregarine. The only possible indications of chromatin are furnished by small dots or condensations at the junctions of the rays with the membrane; they are best seen in iron-hæmatoxylin preparations (fig. 54). We can get no evidence of a red colour at these points after Twort, however, and so do not feel at all certain that they are chromatin.

A correct interpretation of the characteristic central body in the nucleus is best gained from Twort films. Iron-hæmatoxylin films must be very well extracted, and then the same or a similar condition is revealed. But in those iron-hæmatoxylin smears where the whole karyosomatic mass is stained almost uniformly black (as in figs. 53 and 56 for example), it is safe to say that an excess of stain still prevents the details from being apparent. The true structure appears to be as follows: In the centre is a fairly large, clear region, oval or rounded, which is stained grey in iron-hæmatoxylin films and a pale green (distinctly paler than the rays) in Twort preparations. This is the basis or ground-work of the karyosome and is probably of a plastin-like nature. The chromatin is located at the surface, or at any rate in the peripheral region of this plastinoid basis. In the smaller nuclei the chromatin is mostly in the form of granules or small masses of varying number (usually three to five) and size, and more or less separate from each other (figs. 55, 57*a*, 68*a* and *b*); but in the large nuclei we frequently find the chromatin forming a complete zone or ring around the paler area, with thickenings or bulgings here and there (figs. 57, *f* and *g*, 68, *c*, *d*) corresponding to the small masses in the other case.

One important detail remains to be mentioned, namely, the presence of a small, distinct granule in the centre of the plastinoid area, which is probably of constant occurrence. It is readily made out in Twort preparations (cf. fig. 67, 68, *a–f*); sometimes it is green in colour, but in other cases the colour appears to be a mixture of both the red and the green; it is, however, never of the same sharp red colour that the chromatic masses are stained. This granule can be distinguished also in the individuals on iron-hæmatoxylin smears, but not so easily.

Comparing, now, the appearance of the nucleus in Giemsa smears, a condition is generally found which at first sight seems to be diametrically opposite to that shown by iron-hæmatoxylin films—that is to say, centrally or excentrically is a comparatively clear, faintly stained area, while all the rest of the nucleus is stained red more or less deeply (figs. 34, 35, 38, *a–d*), the periphery, in the neighbourhood of the membrane, being perhaps darkest. The clear area corresponds without doubt to the central part of the karyosome, i. e. to the plastinoid basis free from chromatin. Rather curiously, the central granule, referred to above as occurring in the plastinoid part of the karyosome, is often very conspicuous, probably because it is to a certain extent artificially enlarged by the stain. The remarkable feature about these Giemsa-stained nuclei, and the one which creates such a false impression, is that the nuclear sap is often so loaded with stain that not only the rays but also the chromatic zone or ring immediately surrounding the central area is indistinguishable as such. Occasionally, in more favourable pictures, the chromatic zone is more deeply stained than the nuclear sap and can be distinguished somewhat better (fig. 37); and now and then coarse indications of the rays proceeding to the periphery can also be made out (fig. 38, *b*, *c*). Hence we have little doubt that here also the structure of the nucleus agrees really with that above described.

Owing to the scarcity of Trypanosoma triglæ in our preparations the few individuals present on wet films do not show the nuclear structure very satisfactorily. Extraction had to be carried on quite in the dark, as it were, and neither in the individual drawn in fig. 51 from a film stained with iron-hæmatoxylin, nor in that of fig. 65, from a preparation stained with Twort, has the extraction been carried far enough. From these two examples, however, it is quite obvious that the nucleus is of the same karyosomatic type, and fig. 51 affords indications that the structure of the karyosome itself is similar to that above described.

We regard the above instances as indicative of the typical character, speaking broadly, of the nucleus of a hæmogregarine and the trophonucleus of a trypanosome.

So far as the case of the trypanosome is concerned, it is already quite evident, from figures published during the last year or two, since the use of the iron-hæmatoxylin stain became more general, that the trophonucleus is in the main constituted on the same plan, having most of its chromatin associated with a definite karyosomatic body. Besides Miss Robertson (**17**), Minchin has shown this to be the case both in various fish-trypanosomes (**13**) and also in T. lewisi (**14**). We may mention that during the progress of our work at Rovigno we have obtained a similar result in the case of the trypanosomes in the little owl; and we see that recently Rosenbusch has published figures (**20**) of cultural forms of these trypanosomes (which he calls "Hæmoproteus noctuæ" and "Leucocytozoon ziemanni") showing this same nuclear structure.

Further, we are inclined to think that in many cases the minute details of the karyosome will be found to be similar, that is to say, as regards the tendency of the chromatin to be located at the periphery of the plastinoid basis, and the presence in the central, clearer zone of a definite granule. Of course, nuclei with a large karyosome may be expected to show this more distinctly than those with a very small karyosome (such as, for example, T. lewisi.) One of us (H. M. W.) has several times observed, in Giemsa preparations of a trypanosome of the chaffinch,[1] an appearance of the nucleus quite similar to that in fig. 37, namely, a conspicuous granule occurring in the centre of a clear zone in the middle of the nucleus; and the interpretation of the whole appearance is doubtless also the same. It is interesting to note that some years ago Laveran and Mesnil, in their account of certain trypanosomes of fishes (7), published a figure of T. remaki, of the pike, which showed the same nuclear appearance. We remember thinking this unusual at the time, as it was quite different from the uniform granular character which the nucleus was generally described as possessing.

These finer details of the karyosome are best revealed, perhaps, by Twort; in the case of films stained with iron-hæmatoxylin, the stain must be very well extracted, or else the whole karyosomatic mass is too heavily stained for them to be made out. This is evident by comparing our various figures. From many of the figures in the above-mentioned papers we should say that extraction in those cases had not been carried far enough for this purpose. For instance, in most of the resting nuclei of the various trypanosomes drawn by Rosenbusch (l.c.), the karyosome is too dark to show the central granule.

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[1] It is hoped to publish an account of this parasite, and of others in the chaffinch, very shortly.

There can be no doubt that this granule or centriole is the intra-nuclear centrosome first described by Schaudinn in the trophonucleus of his trypanosome in the little owl. It is also evident that it acts as a division-centre, and forms an intra-nuclear spindle at the commencement of nuclear division. This phase is well shown by several of Rosenbusch's figures. Again, to compare a dividing stage described from a Giemsa-stained preparation, Minchin, in his account of T. grayi (**11**), figures an intra-nuclear granule at each end of the spindle still connecting two daughter-nuclei, immediately after division has taken place. Hence this intra-nuclear centrosome[1] is doubtless a regular constituent of the trophonucleus of a Trypanosome.

It will be noticed from our figures that, in the Giemsa-stained preparations of T. raiæ, the red-stained part of the nucleus is fairly uniform or homogeneous in appearance. It is more usual, however, for the nucleus of trypanosomes stained by the Romanowsky method to appear granular in structure, apparently consisting of small or medium-sized granules in close contact, and forming a compact mass (cf. the selected figures, either in the article on "Trypanosomes" in Lankester's 'Treatise on Zoology,' or in Lühe's article in Mense's 'Handbuch der Tropenkrankheiten'). This appearance is quite easily capable of explanation when the known tendency of the Romanowsky stain to be deposited in excess around anything of a chromatic nature is borne in mind. We may suppose that in such cases there is a certain amount of chromatin distributed in the nuclear sap or karyolymph (in addition to that associated with the karyosome); this is most probably in the form of very fine granules, which are of course magnified by the stain to many times their real size. Hence the effect is produced of a granular mass, such as has been so often described. By this means the clear central area, indicating the position of the karyosomatic body, is generally occluded completely and indistinguishable.

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[1] Moore and Breinl (**14A**) use the term "intra-nuclear centrosome" in a different sense from ourselves, namely, for the entire central body which we regard as the karyosome. They do not seem to have distinguished at all the centriole contained in the karyosome.

We have now to consider, briefly, the hæmogregarine-nucleus. Here, too, there can be no question but that the true nuclear structure is better revealed by stains like iron-hæmatoxylin and Twort than by the Romanowsky method of staining. Nearly all the illustrations of hæmogregarines which we have seen are from parasites obviously stained by the latter method. Prowazek, it may be mentioned, in his paper on H. platydactyli (**16**), has three figures which were drawn from preparations stained by Grenacher's hæmatoxylin, and these also give indications of the same type of structure—an irregular reticulum carrying chromatin-grains and masses of various sizes—which we have found in H. rovignensis. The nuclei in these figures of Prowazek's differ very greatly from those he has drawn from Romanowsky preparations; many of the latter, we are convinced, do not give at all an accurate idea of the nuclear constitution.

Of all the other figures of hæmogregarine-nuclei at which we have looked, those of Börner, in his account some years ago (**4**) of reptilian hæmogregarines, seem to convey most approximately the true idea of the nucleus. From his "Tafelerklärung" we gather they were drawn from Romanowsky preparations; but for this intimation we should have regarded them as from preparations stained by some hæmatoxylin method, both from the appearance of the nuclei and from the manner in which the figures are coloured. We are inclined to think the nuclei in some of his figures may be possibly a trifle schematic both as regards the uniform size of the granules and the rather suspicious regularity of the reticulum; but in many of the other figures there is, in our opinion, an indication of the nuclear structure which is probably as correct as it is possible to obtain it by the Romanowsky method. In none of Börner's figures is there anything remotely resembling a karyosome, and, in fact, the author expressly mentions that he never observed such a structure in the nucleus.[1]

Numerous figures accompanying the description of new hæmogregarines have been published during recent years, all of them naturally from Romanowsky preparations. It would take too long to cite them; nor is it necessary. It is sufficient to say that in no case can the structural details of the nucleus be deciphered. In all cases it is obvious that the nucleus drawn was still hopelessly overloaded with stain. At the best the nucleus is figured as a dense granular mass, bearing often a strong resemblance to those in our figures from Giemsa-stained preparations, from which it may be inferred that its structure conforms to that of H. rovignensis and to what we consider is the general plan. At other times nothing but a "splotch" of colour, from which it is impossible to ascertain anything, is depicted.

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[1] The only instance of which we are aware, where anything resembling a karyosomatic nucleus appears to be present, is in certain figures of Wenyon's (**22**) on Pl. 12, purporting to represent H. gracilis in the liver of Mabuia. The figures are from preparations stained by hæmatoxylin. We think it most likely that Wenyon has figured besides phases of a hæmogregarine, also phases of a coccidian, the latter being the ones in which the nuclei show a karyosome. His fig. 29 shows undoubtedly the development of typical merozoites of a hæmogregarine (cf. H. simondi); and it is only these merozoites which he figures also in the red corpuscles. His figs. 27, 22, and 31, on the other hand, we consider, represent a Coccidian; the two latter especially appear very like young coccidian schizonts. Since our MS. was sent to the printers the memoir by Hahn (**4B**) has appeared. We can only point out here that Hahn uses the term "karyosome" in a sense quite different from that in which we understand the word, namely, to mean the entire nucleus when in a condition "devoid of visible chromatin bodies" (p. 331). He terms such bodies "achromatic nuclei" (which seems to us rather a contradiction in terms), and calls them "karyosomes, in the sense that they are the bodies from which the chromatin bodies subsequently arise."

In conclusion we have only to point out that it seems clear that the nucleus of a hæmogregarine is of a very different type from that of a trypanosome. The former is characterised by its chromatic reticulum, with chromatin grains or masses more or less generally distributed upon it. In the latter the greater part or nearly all of the chromatin is, as it were, condensed around a plastinoid basis, the whole forming the conspicuous karyosome; and in the centre of this plastinoid area is a definite granule, the intra-nuclear centrosome.

So far, therefore, as the hæmogregarines at least are concerned, we are totally unable to agree with Hartmann (4c), who proposes to remove the Hæmosporidia entirely from the Sporozoa, and place them with the trypanosomes and their allies among the Flagellata as a group named Binucleata.

Postscript.

We had not intended to refer in this paper to the nucleus of Halteridium. Quite by chance, however, we have noticed a couple of sentences at the end of Berliner's account of the cytology of certain Flagellates (1), which relate to the structure of Halteridium noctuæ and Leucocytozoon ziemanni, as shown by iron-hæmatoxylin. There is no reference to this point in the title or list of contents, and we have only had our attention drawn to Berliner's figures since our present paper was completed. We refer to Berliner's note because we have ourselves obtained similar indications of the nuclear structure of these parasites during our work at Rovigno. We will only point out here that from Berliner's published figures, and equally from our own preparations, there can be no doubt that the nuclear structure of Halteridium is quite different from that of a hæmogregarine, and, on the other hand, remarkably like that of the trophonucleus of a trypanosome, in being of the karyosomatic type. Further, Berliner mentions and figures the occurrence at times of a distinct extra-nuclear granule, connected by a fibril with the main nucleus, which he regards as representing the kinetonuclear element of a trypanosome perhaps in a somewhat reduced ("rückgebildet") condition, consequent on the intra-cellular, "resting" condition of Halteridium. We are very pleased to have this independent confirmation, and from iron-hæmatoxylin preparations, of the occurrence of nuclear dimorphism in Halteridium, a feature which one of us (H. M. W.) has already described (**23**) in the case of another species parasitic in the chaffinch, though unfortunately in this instance only Giemsa-stained smears were available. There can be little doubt, therefore, that Halteridium, in regard to its nuclear structure at all events, shows very much closer affinity to the trypanosomes than do the hæmogregarines.

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EXPLANATION OF PLATES 8–10,

Illustrating Professor E. A. Minchin's and Dr. H. M. Woodcock's paper on "Observations on certain Blood-parasites of Fishes occurring at Rovigno."

[The drawings on Pl. 8 are all from Giemsa-stained preparations, those on Pl. 9 are from films stained with iron-hæmatoxylin, and those on Pl. 10 from films stained by Twort's method. All the figures relating to Hæmogregarina rovignensis are magnified 3000 times linear; those relating to Trypanosoma triglæ and T. raiæ 2000 times.]

PLATE 8.

Figs. 1–28.—Hæmogregarina rovignensis. Figs. 1–11 are from the first infected gurnard, figs. 12–27 from the second, and fig. 28 from the third infected fish. Figs. 5–11 are from a very thin smear, which was faintly stained; both corpuscles and parasites are uniformly flattened out, but the nuclei of the parasites come out better than in any other Giemsa-stained smears.

Fig. 1, 2, 5–9, 16, 17.—Ordinary small forms, schizonts.

Fig. 3.—A small form, free from the blood-corpuscle, occurring in a smear from the kidney.

Figs. 4, 10, 18–21.—Large forms of the slender or male type.

Figs. 11, 22–26.—Large forms of the broad or female type.

Figs. 12, 13.—Young individuals of the slender, male type.

Figs. 14, 15.—Young and intermediate-sized individuals respectively of the broad, female type.

Fig. 27.—A double infection of the corpuscle, with two small forms of the parasite.

Fig. 28.—Small form showing a constriction of the nucleus in the middle.

Figs. 29–31.—Trypanosoma triglæ.

Figs. 32–37.—T. raiæ. Fig. 33 is of a small parasite, fig. 35 of an intermediate-sized one; the rest are of large individuals.

In fig. 34 the small vacuolar spaces were originally occupied by black grains similar to those seen in the parasite of fig. 37.

Fig. 38, *a–d*.—T. raiæ, trophonuclei of different parasites (× 3000).

PLATE 9.

Figs. 39–50.—H. rovignensis. Figs. 39–43 are from the first infected fish, figs. 44–50 from the second one.

Figs. 39–43, 46, 47.—Ordinary small forms (schizonts).

Figs. 44, 45.—Young and intermediate-sized individuals respectively of the slender or male type.

Figs. 48, 49.—Large forms of the broad, female type.

Fig. 50.—Large individual of the slender, male type.

Fig. 51.—Trypanosoma triglæ.

Figs. 52-56.—T. raiæ.

Fig. 55.—The trypanosome of this figure is on a different film from the others, one from which the stain has been considerable more extracted.

Fig. 57, *a–i*.—T. raiæ, trophonuclei from various trypanosomes; *e* and *h* are from large trypanosomes, the rest from smaller or intermediate-sized parasites (× 3000).

PLATE 10.

Figs. 58–64.—H. rovignensis. Fig. 58 is on a film from which the red part of the stain (neutral red) has been much less extracted than in other cases.

Figs. 58–61.—Ordinary small forms.

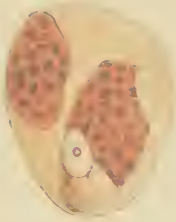
Figs. 62, 64.—Large broad forms.

Fig. 63.—Large slender form.

Fig. 65.—T. triglæ.

Figs. 66, 67.—T. raiæ.

Fig. 68, *a–f*.—T. raiæ, trophonuclei from various individuals; *d–f* from large parasites (× 3000).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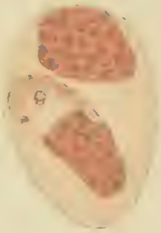
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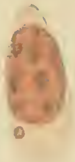
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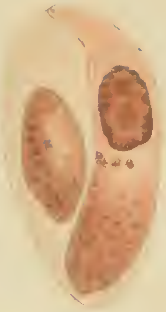
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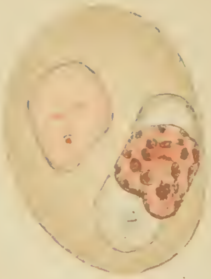
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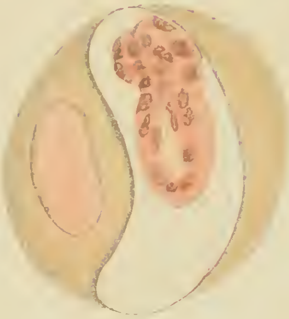
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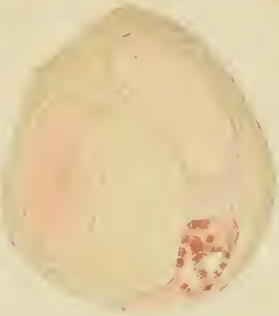
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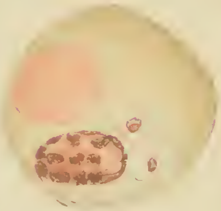
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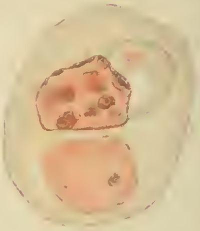
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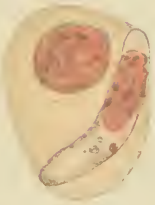
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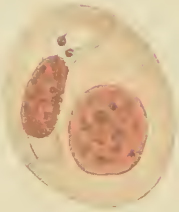
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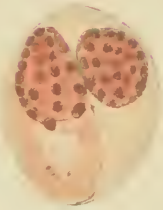
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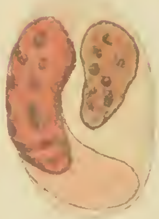
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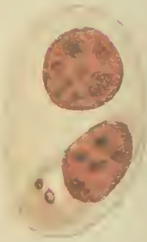
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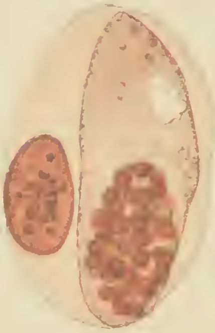
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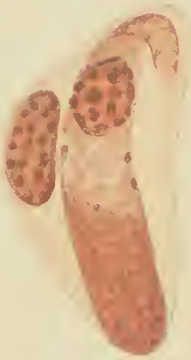
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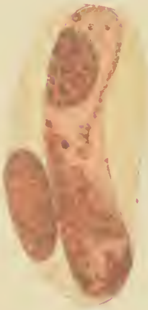
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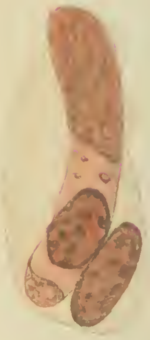
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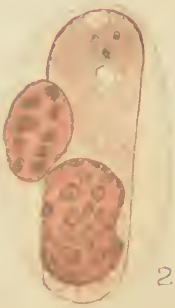
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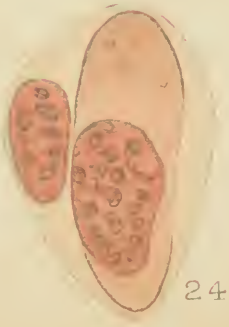
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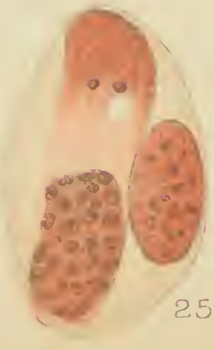
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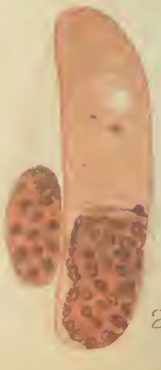
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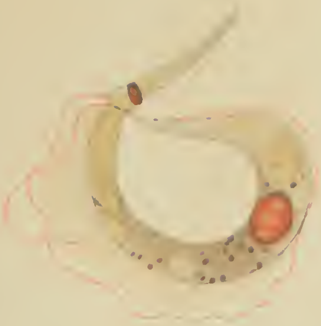
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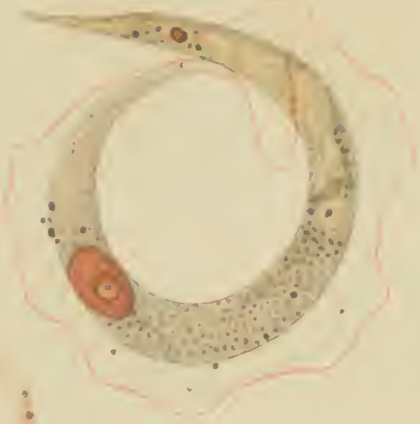
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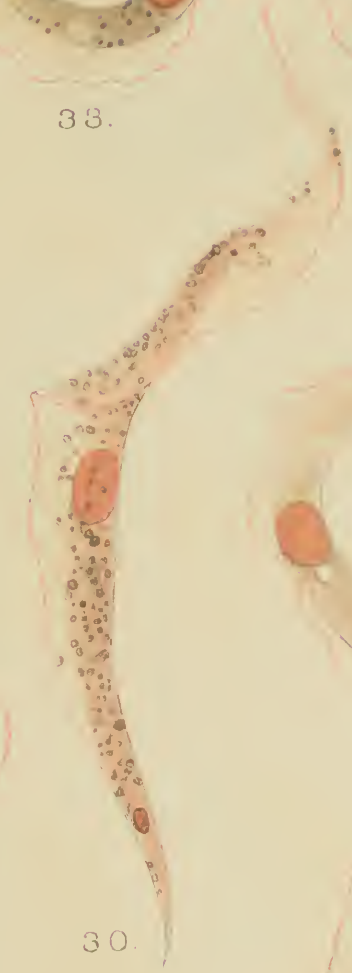
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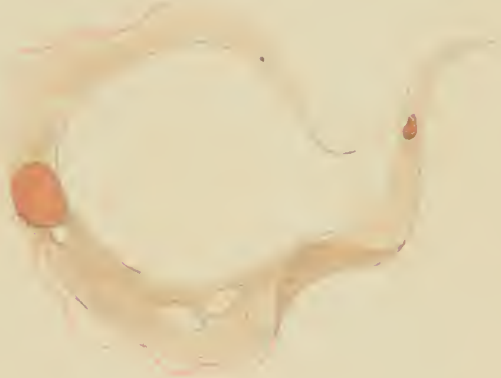
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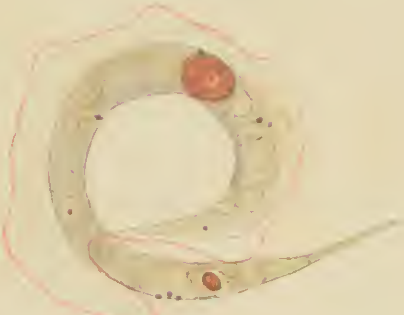
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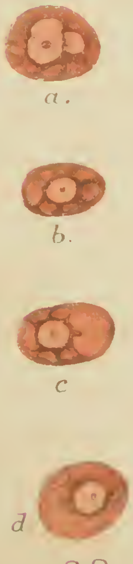
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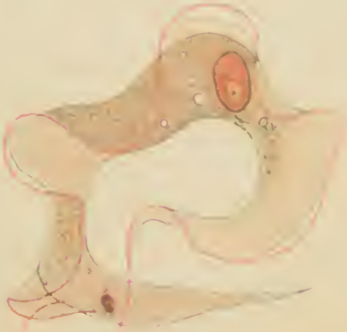
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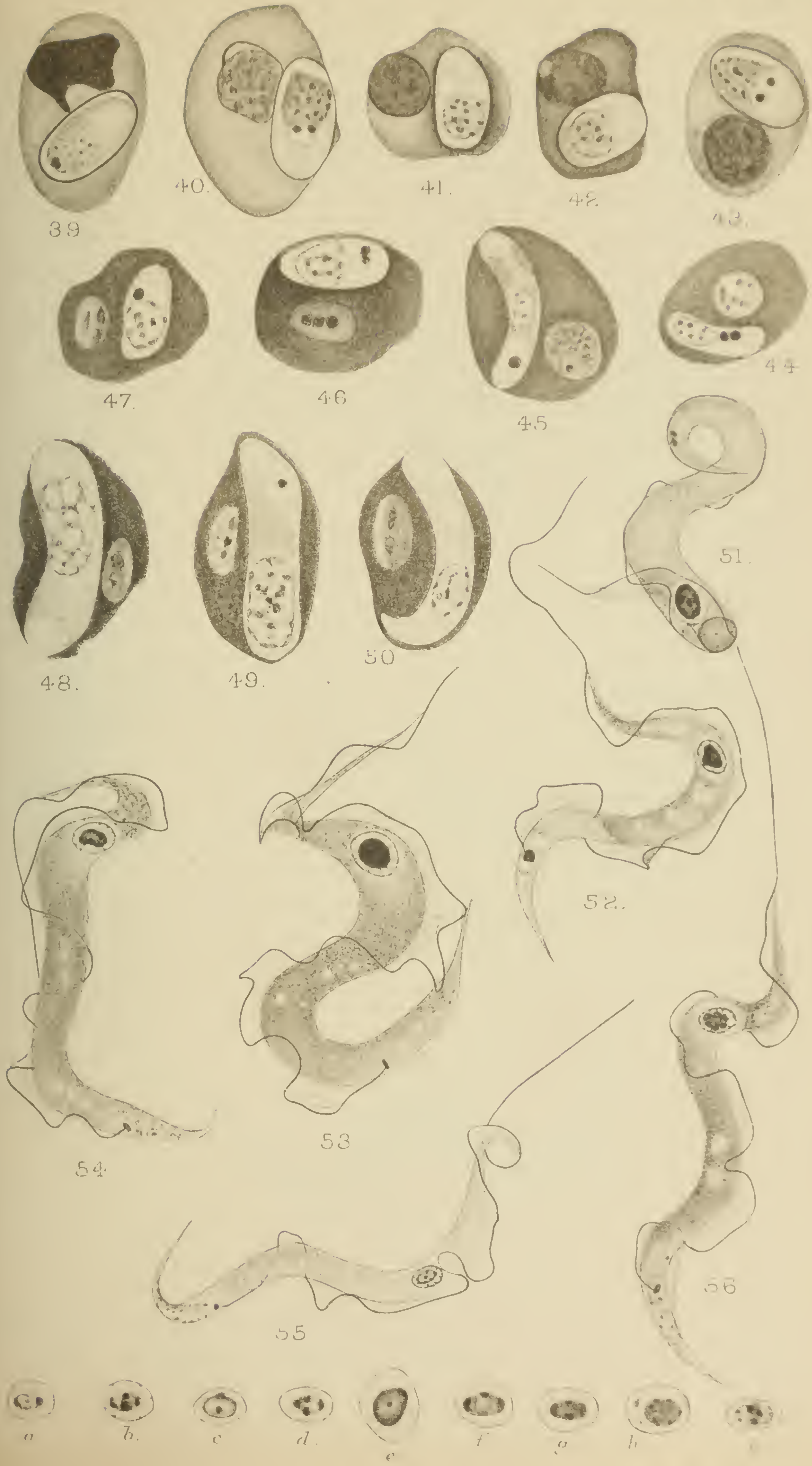
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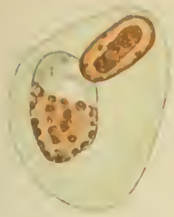
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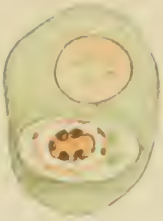
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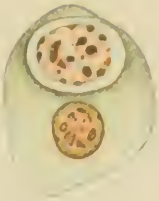
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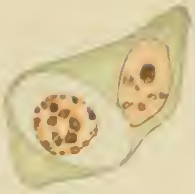
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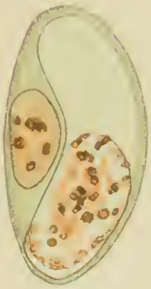
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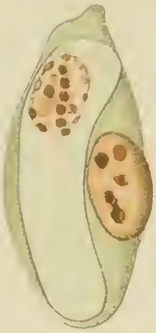
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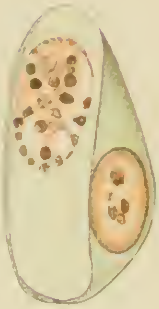
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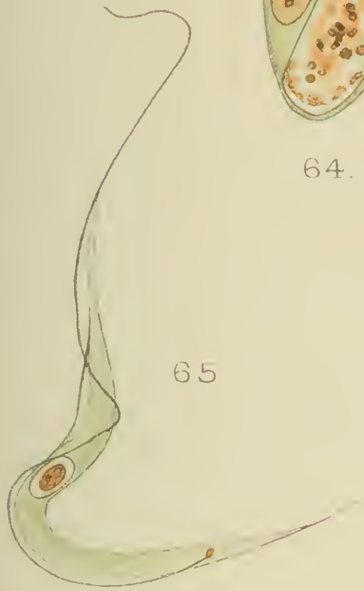
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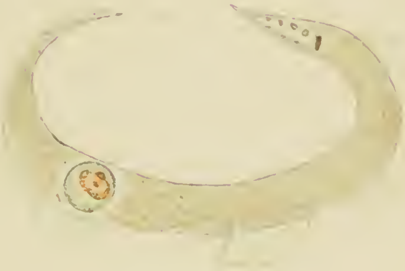
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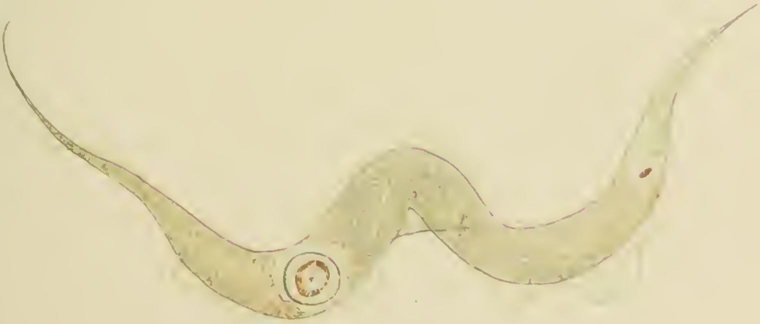
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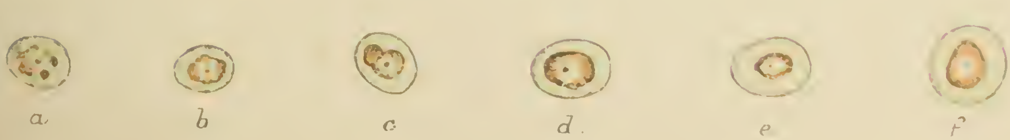

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THE CULTURAL CHARACTERISTICS OF THE MICROBACILLUS OF ACNE.

By E. H. MOLESWORTH, M.B., Ch.M.Sydney.

(From the Lister Institute of Preventive Medicine, and the Dermatological Department, King's College, Hospital.)

The object of the present research on the microbacillus of acne was to reconcile, if possible, those accounts of its biological and cultural properties which have been given by recent contributors to the subject, with those originally described by Sabouraud in 1897. Such work is rendered all the more necessary when it is realized how great are the differences in the pathogenic qualities ascribed to this bacillus by the various authors who have treated the subject.

In order to bring these discrepancies of opinion clearly before those interested in the subject it is necessary briefly to refer to the principal works dealing with the acne bacillus.

It was Unna[1] who in 1896 discovered this organism in the comedo, but who, being unable to cultivate it, drew, from purely histopathological data, the conclusion that it was the cause of the comedo and the subsequent suppuration. The classical work, however, upon the subject is that of Sabouraud,[2] who, studying the organisms both in the lesion and in culture, was able to speak more authoritatively on the subject. He assigns to the microbacillus the causative agency in the production of the comedo, but decides that the suppuration is due to the staphylococcus. This opinion he supports, first, on the histological grounds that the staphylococci are found in the phagocytes, while he was never able to find microbacilli undergoing the process of phagocytosis in these cases; secondly, because he was never able to produce suppuration even by injecting huge quantities of microbacilli under the skin. For the cultivation of the microbacillus a special acid glycerine agar was required, and the details of its preparation will be found in his work. The inoculation was made with the central portion of a seborrhoeic filament, which, by the aid of a lance-headed needle, was carried into the medium to a depth of several centimetres. Incubation at 37° C. results in rapid growth of staphylococci of the non-liquefying variety, followed in four days by the appearance of a red-brown button, which gradually increases

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until it reaches the size of a small currant. This is the culture of microbacilli, which can be obtained pure by reinoculating after a month's growth, at which time the staphylococci are found to have died. He never succeeded in obtaining an ordinary stroke culture, but obtained subcultures by several other methods—for example, by inoculating and incubating eggs. These researches were performed at a time when anaërobic methods were very imperfect. Inoculation experiments on animals were uniformly negative in Sabouraud's hands.

These results were confirmed by Gilchrist,[3] but it is extremely doubtful whether the organism he was dealing with was identical with that of Sabouraud. Cultures of his bacillus were fatal to guinea-pigs and white mice in some cases.

In this state the matter rested for some years, the conclusions of Sabouraud being fairly generally accepted in France and England.

Then followed in 1907 some work on the sebaceous glands by Hallé and Civatte,[4] who, in making anaërobic cultures from comedones, found constantly colonies of an anaërobic organism which corresponded very closely in microscopical characters with the organism seen in the comedo. This they pointed out to Sabouraud, who, with Hallé, compared the organisms obtained by both methods, and found by inoculating one of Sabouraud's cultures in a deep glucose-agar tube by the stroke method (which Hallé and Civatte, pupils of Veillon, used), that Sabouraud's organism grew only anaërobically. This is a surprising fact, the only explanation for which is that the microbacilli in the centre of the material used for inoculation in Sabouraud's cultures were so protected by the surrounding fatty material that the penetration of oxygen was insufficient to prevent growth. At the present moment Sabouraud has only modified his previous teaching in so far that he impresses the fact that the microbacillus is anaërobic in culture.

The next contribution to the subject was that of Fleming,[5] who, in describing his cultural experiments, came to some conclusions at variance with those of Sabouraud. In the first place, he describes pure cultures of microbacilli by surface inoculation of pus from cases of acne, on a medium described by him in his work. This fact and the failure to grow staphylococci in all cases, led him to the opinion that the latter when present were only the result of accidental contamination. Accordingly he makes the microbacillus the cause of both comedo and pustulation—a conclusion supported by his success in obtaining a pustular eruption by rubbing a culture into the arm of a susceptible person. This Sabouraud had failed to obtain. Secondly, he described his cultures as being made by ordinary surface inoculation, by which method Sabouraud always failed to get growth. Results of calculation of opsonic indices and the therapeutic action of vaccines prepared from the organisms cultivated were also given in support of his views.

This paper evoked criticism from Whitfield,[6] who maintained his previous opinion that suppuration in acne was due to staphylococci by reference to histological and cultural experiments, and could not agree that the results of opsonic work justified the conclusions drawn by Dr. Fleming.

Quite recently a paper by Südmersen and Thompson[7] appeared recommending the use of a strongly acid agar with the addition of horse serum, but the most significant part of the work was their recommendation of anaërobic methods for the cultivation of the organism.

Thus as the matter stands at present the microbacillus is described as being aërobic and pyogenic by some, anaërobic and non-pyogenic by others, and a multitude of minor differences in characters morphological and cultural are disputed.

My own personal work consists of a series of primary cultures from cases of acne, now totalling over 30. The materials used for inoculation were generally comedones, sometimes seborrhoeic filaments and pus.

The medium employed for these cultures was simply 2 per cent. glucose agar in deep shake tubes or stabs, or slopes incubated anaërobically. Personally I find the most convenient method for isolating pure cultures the deep shake method as recommended by Veillon. The agar is melted and allowed to cool to about 45° C., when a drop of an emulsion is added, made by crushing a comedo, or such material as is to be used, in a few drops of salt solution, mixing well and transferring to a second tube 3 or 4 drops from the first, and 10 to 20 drops from the second to a third. The agar is allowed to solidify and the tubes incubated at 37° C. By this method in the second and third tubes isolated colonies are obtained and can be easily picked out for subculture. This is most easily done by using a sterile Pasteur pipette drawn out to about 1·5 mm. in lumen. To this a rubber tube with a mouthpiece at one end is attached, and when the tube is held horizontally against the light the pipette can be pushed down to the colony, which then is sucked up into the lumen, withdrawn, emulsified, examined, and re-inoculated by a procedure exactly similar to that described above.

In cultures made from comedones or pus by this method staphylococcic colonies appear in a day or two, chiefly confined to the upper aërobic areas of the first tube; but after an interval of four days minute colonies appear in myriads, confined to the anaërobic areas. These slowly increase in size, and when discrete assume a lenticular form or often a shape composed of regularly intersecting lenticular bodies; the colour of these colonies is greyish-white. Scattered amongst these white growths, especially if the material inoculated has been a comedo, are seen a few similarly shaped colonies, but red and larger in size.

On microscopic examination the organism found in the white colonies is seen to be a short plump Gram-positive bacillus, exactly corresponding in appearance with the organism seen in a smear of the comedo The red colonies show cocco-bacillary forms also Gram-positive, but not recalling the organism found in the comedo. Subcultures made by the shake method readily show the anaërobic character of both these organisms which only grow at a distance not less than 1 cm. from the surface.

Stab cultures made in the usual manner in glucose-agar tubes give growth in the deeper parts of the stab, but show no special characters. On slopes inoculated from a pure culture of the microbacillus, incubated in a Bulloch's apparatus or Buchner's tube, isolated whitish colonies or a continuous growth is obtained according as a dilute or thick seeding is made. By these surface inoculations one obtains longer and more diphtheroid organisms with occasional irregular forms, contrasting with the uniform short plump forms of the mother colony.

Cultures may also be obtained in glucose broth incubated anaërobically, but a large amount of material must be inoculated. On ordinary agar, and in ordinary broth, attempts were almost uniformly futile, or such an insignificant growth occurred that one doubted whether the seeding had increased at all. For a very long time attempts to obtain an aërobic culture failed, but finally, by using a very old strain frequently subcultivated, an aërobic growth was obtained. This is more likely to succeed when material from a slope culture is used than when a colony from a deep shake tube is inoculated.

Similar methods are successful in cultivating the organism growing in red colonies, but the colour production soon diminishes on subcultivation, and is not evident on slopes.

To compare this organism with a culture obtained from Sabouraud was the next step. In this I quite confirm the results obtained in Paris, an emulsion from Sabouraud's culture inoculated by the shake method growing only in the anaërobic areas. Also, I was able, after some difficulty owing to the lack of sticky coherence required for this method of inoculation, to transfer a colony from an original culture in a shake to Sabouraud's medium in the manner described by him. The mass increased and assumed a brownish hue as seen in Sabouraud's culture.

These cultures have been accepted by Sabouraud as identical with the organism described by him, but a culture of Fleming's organism when shown to him was not recognized as being identical with the microbacillus. With regard to animal inoculations made with the microbacillus, negative results with guinea-pigs were always obtained. The morphological and cultural characters of Gilchrist's bacillus, and its marked pathogenic qualities for guinea-pigs and white mice, lead one to think that his organism was more probably a form of streptothrix, and not the microbacillus of acne. In the human subject no result was obtained by rubbing in large quantities of the culture.

In comparing the organism isolated by anaërobic methods with two strains obtained from Dr. Fleming and a third obtained through Dr. Eyre of Guy's Hospital, a considerable number of important differences was found. First, and most important, it was found that Dr. Fleming's organism was strictly aërobic, growing only in the uppermost centimetre of a shake or stab culture, and never in the depths. On slopes incubated anaërobically no growth was obtained. Secondly, his organism grew quite readily, and appeared in about forty-eight hours on ordinary agar, and in ordinary broths, on which media the microbacillus, even under anaërobic conditions, is at least extremely difficult to cultivate. Even on glucose agar four days elapse before the colonies of microbacillus become evident. It was on these characters that cultures shown to Sabouraud were rejected by him.

About two months ago, however, I obtained a fourth strain from Dr. Fleming, and, much to my surprise, found that it grew anaërobically, being, in fact, exactly similar to those growths obtained by educating the microbacillus to aërobic growth. This culture is undoubtedly different from the others, and is probably the true microbacillus

In drawing conclusions from this work we are bound to consider, first, whether the organism cultivated by Dr. Fleming was identical with that grown by Sabouraud. I am forced to the opinion that this is not the case, since it is inconceivable that an organism really an anaërobe should be so changed in its characters by cultivation under anaërobic conditions as quite to lose the power of growing anaërobically. And even a very old strain of microbacilli which has been educated to aërobic conditions will, if transferred to anaërobic conditions, immediately reassert its preference. The early appearance of growth in Fleming's cultures, which is always less than the four days necessary for the first sign of microbacilli in cultures, is also a point against the identity. Again, the pustular eruption described by Fleming on inunction of a mass of his organisms into the arm of a susceptible person is not obtained by a similar experiment with Sabouraud's organism. Now, since it is admitted by all who have compared the two, that the organism seen in Sabouraud's cultures and that obtained by ordinary anaerobic methods are identical, there is no reason again to distinguish between Fleming's organism and the anaërobe.

The next question for consideration is, Which is the real microbacillus of acne?

In the first place, Fleming's organism is admittedly difficult to obtain, and a culture has not been forthcoming in my hands or in those of Dr. Whitfield, in spite of numerous essays. On the other hand, the colonies obtained by anaërobic cultivation are in myriads, as would be expected when one remembers the vast numbers of the organisms in the comedo. Again, Dr. Fleming does not mention a single instance of successful cultivation of his organism from comedo, all his cultures having apparently been made from pus, which is more liable to contamination and not so rich in the organism whose cultivation is sought as the comedo.

Most of Dr. Fleming's conclusions seem to have been based upon the opsonic indices of his patients to the organism cultivated. But it seems to me unjustifiable to pronounce an organism the cause of a particular lesion on these grounds. There are at least other possibilities.

With regard to inoculation experiments, at first sight the results published by Dr. Fleming appear more promising, but it is to be remembered that comedo formation and not suppuration is the essential lesion of acne vulgaris, and no account of a production of comedo is given in the description of his results. In addition, treatment with vaccines of Fleming's organism apparently caused increase of the amount of pustulation; but no mention is made of the disappearance of comedo under such treatment.

The inoculation experiments by inunction with the anaërobe are, it is true, unconvincing. But nearly everybody is at least seborrhoeic, and the liability of the subject to react to the presence of the organism by comedo production seems to be limited, in the vast majority of cases, to the age of puberty and early twenties, and then to certain well-defined localities—for example, face and back where inoculation results would be open to question. Those who have suffered from acne in their teens appear, as a rule, to become immune to the organism to the extent that the only reaction observed is a persistent excessive fat secretion.

Next, as to the causation of the pustulation, on only one occasion did I fail to find staphylococci in culture from pus, though it is only fair to acknowledge that Sabouraud in his vast experience has occasionally found cases which gave no staphylococci. It is true also that microbacilli can be cultivated in great numbers from the pus, but not more than might be expected considering the amount of comedo débris present in this material.

In conclusion, a great deal has been written about media for the cultivation of this organism. In my opinion the complicated agars recommended are quite unnecessary. The secret of success in cultivating the organism is the use of anaërobic methods. Under these conditions a simple and easily prepared medium like 2 per cent. glucose agar gives luxuriant growth. Südmersen and Thompson, who also used anaërobic conditions, recommend a highly acid medium (+ 40 to phenolphthalein). But even by reducing the acidity to + 20, I was unable to obtain a material which was sufficiently stiff to be sloped.

Dr. Sabouraud still uses his special medium and method of inoculation, but for ease and rapidity of obtaining a pure culture from all sorts of material, I think it must be admitted the anaërobic methods are the most satisfactory.

In conclusion, I wish to express my gratitude to Dr. Ledingham at the Lister Institute for his goodness in controlling my work and advising lines of action; to Dr. Whitfield for the material he provided, and assistance throughout the work, and especially to Dr. Sabouraud for his kindness in furnishing me with the full literature on the subject, and in examining and identifying my cultures.

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OBSERVATIONS ON THE EVOLUTION OF IMMUNITY IN DISEASE.

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I. Introduction.

The following experiments constitute an attempt to follow out in detail the stages by which immunity is established, in the course of a generalised bacterial infection (Pseudotuberculosis of rabbits). Two aspects of immunity are considered, firstly the presence, in the circulating fluids, of specific antibacterial substances, and secondly the power of rapidly producing such substances in answer to the specific stimulus. That is to say, attention is directed, not only to the quantity of specific antibodies present on any day of the disease, but also to the response which the animal can make to various doses of bacterial vaccine. For the immune animal is both more vigorous and more sensitive than the normal, in its reaction to a renewed dose of poison (Wassermann and Citron, 1905). Naturally the facts established with regard to one disease only, cannot be predicated at once of other diseases, in other animals. Still it is hoped that the systematic study of one disease may give some help in coordinating the large but somewhat disjointed mass of clinical observation which is already available.

It is not a new observation that, in the course of the severest bacterial infections, the early days are marked by the absence of any specific reaction on the part of the patient. The general inflammatory reaction is present but no specific antibodies are formed. After a shorter or longer period of delay, more or less immunity is developed. One may therefore distinguish two stages in the course of an infection.

(*a*) A period of inertia, as regards the formation of antibodies.

(*b*) A period of immunising reactions.

Pneumonia affords a good example of this distinction. J. G. Macdonald (1906), working with Bulloch, showed that, in this disease, the opsonic power of the blood remains low for about a week after the onset. At the end of this period, there occurs a decided rise in the amount of pneumococco-opsonins, which is followed regularly within a few hours, by the crisis. Wolf (1906) obtained similar results. In other diseases the onset of an immunising reaction may be neither so sudden nor so decidedly effective, yet the two stages may be recognised, as in the case of enteric fever. It is well known that the agglutinating power of the serum, which marks one of the specific reactions of the organism to this infection, is generally delayed in its appearance till about the tenth day. Malta fever offers another example of such delay, as in this disease the agglutination reaction is not obtained before the fifth day, and may remain absent till a much later period[1]. Reference may also be made to the streptococcal infection which seems to be a constant feature of scarlet fever. It was shown independently in America and England, by Hektoen (1907) and Banks (1907) that, in the first stages of this disease, the streptococco-opsonic index of the patient was generally low, but that a rise occurred in connection with defervescence, though not before the fourth day. Long continued lack of any immunising reaction has been observed by Wright (1907) in various septicaemic conditions, such as streptococcal endocarditis and Malta fever. The same paper shows how the opsonic immunity produced in response to such infections may be fluctuating and inefficient when it has at last arrived. These results in so far as they relate to Malta fever are confirmed by Bassett-Smith (1907).

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[1] Aldridge (1898), Bert and Lamb (1908), Durham (1898).

In order to examine into the production of these phenomena more closely, experiments were made on the following lines:

The opsonic response following the inoculation of killed cultures into normal rabbits was investigated, and it was found that different tissues possessed different powers of response to these inoculations. Then, the effect of injecting living cultures into normal rabbits was determined, and after this the effect of inoculations of killed cultures into previously infected rabbits. The reaction of the infected rabbits towards inoculations of killed cultures was found to be profoundly altered during the course of the disease. A further examination was then made of the respective activities of living and killed cultures, in exciting a response in rabbits already infected with the disease, and recovering from this infection.

After describing these experiments, the present paper concludes with a discussion of the factors which influence the immunising reactions, which are observed in the course of the experimentally induced disease. The starting point and groundwork of these observations is provided by the work of Sir A. E. Wright and his fellow-workers, to which it is a pleasant duty to acknowledge my indebtedness.

II. GENERAL DESCRIPTION OF THE EXPERIMENTAL DISEASE.

The infective organism chosen was the *Bacillus pseudotuberculosis rodentium*, which is nearly related to that of plague (*B. pestis*). This bacillus is easily regained in pure culture from the blood of a rabbit or guinea-pig dead of the disease. Its discrete, opaque-white, colonies with crenated margins, are readily recognised; and the absence of contamination, after passage, was confirmed by the sugar reactions.

In the earlier part of the work a strain (designated P) was used which was obtained from the Pasteur Institute, early in 1907; more lately

I have used a strain which was kindly sent me by Dr W. E. Marshall from the Lister Insitute in May 1908 (Strain M).

TABLE I.

Sugar reactions of B. pseudotuberculosis rodentium.

	Reaction	
Sugar	24 hours	48 hours
Lactose	—	—
Glucose	Acid	Acid
Maltose	Slightly acid	Acid
Mannite	Acid	Acid
Dulcite	—	—
Cane sugar	—	—
Inulin	—	—
Sorbite	—	—

The bacillus grows readily on ordinary agar, and an emulsion of bacilli washed off a 24 hours' agar slope culture, by means of broth or saline solution, formed the infective material for inoculation. A measured fraction of the emulsion obtained from one such culture was inoculated directly into the peritoneal cavity of the experimental animal. In rabbits the gravity of the infection may be determined almost at will, by varying the dose between $\frac{1}{100}$ of a culture, which dose the large majority of rabbits will survive, and $\frac{1}{5}$ which constitutes an almost certainly fatal dose. For guinea-pigs $\frac{1}{100000}$ of a culture was found to be an almost certainly fatal dose, death occurred between the 5th and 14th days. Using larger doses, up to $\frac{1}{30}$ of a culture, the fatal termination was found to arrive with considerable punctuality on the 6th or 7th day.

The intraperitoneal inoculation is followed by a general peritonitis, with a small quantity of turbid exudate. After two or three days the whole surface of the peritoneum, both visceral and parietal, is found to be studded with minute grey nodules, and the omentum is thickened and infiltrated. At this stage very few bacilli are to be found in the peritoneal fluid, but smears made from the peritoneum or omentum show large numbers of bacilli, for the most part contained within large mononuclear endothelial cells. Very soon the infection spreads, and small abscesses are formed in the spleen and liver. The small grey nodules previously noted on the peritoneal surfaces, tend to become absorbed, but some of them increase in size, and go on to the formation of abscesses, or caseous masses. All the abdominal glands become affected, and a large mass is often found at the root of the mesentery of the small intestine, composed of glands which have adhered to one another and undergone caseation.

In guinea-pigs the infection usually spreads to the thorax, where the mediastinal glands are found severely infected, and the lungs show numerous miliary abscesses. At death the blood always gave a pure culture of the bacillus.

The immunising reactions called forth by such an infection consist in, (1) an increase of opsonin, (2) the formation of agglutinin, (3) the formation of precipitin. No bactericidal or antitoxic substances can be demonstrated satisfactorily. It is not clear what importance attaches to the presence of agglutinin and precipitin, but it appears that an increase of opsonin is of real and vital significance in combating the disease (Part V, and Fig. 5). Consequently, in studying the fluctuations of the opsonic index, we are observing a manifestation of immunity which is of real importance in determining the fate of an infected animal. Furthermore, as will be shown below, the opsonic index gives much earlier information of an immunising response, on the part of the rabbit, than do observations of agglutination (Figs. 2 to 6, 15, 16, 18). The experience of Leishman and his colleagues of the Royal Army Medical Corps, in studying the effect of various antityphoid vaccines, also show the value of opsonic determinations in giving early information of the progress of immunising reactions[1]. And again the opsonic index has been used with success as a guide to therapeutic inoculations, so that in following its changes one was progressing along a road already partially explored. For all which reasons, in these experiments attention has been chiefly directed to the opsonic immunity.

III. METHODS.

Estimations of agglutination were carried out macroscopically, in capillary tubes of about 1 mm. bore[2].

Opsonic estimations were made by Wright's method (1903) with the modifications which have been adopted in his laboratory as the result of much experience, since its first publication. I had the opportunity of working at St Mary's Hospital in the winter of 1907—1908, and became well acquainted with the work on which Fleming based his article on the accuracy of the method, and some of the sources of error (1908).

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[1] Leishman (1905, 1908), Harrison (1907).

[2] See Wright (1897), and Wright and Smith (1897).

The corpuscular suspension used, was obtained from my own blood. About ·5 c.c. of blood was drawn from the finger into about 3 c.c. of a 1·5 per cent. solution of sodium citrate, and well shaken. This suspension was then centrifuged, and the corpuscles, after removal of the supernatant fluid, were shaken again with 3 c.c. of a ·85 or 1 per cent. solution of sodium chloride. After renewed centrifugation, and removal of the saline solution, the corpuscular sediment was thoroughly mixed. The whole of this material then afforded a uniform mixture of red corpuscles and leucocytes, which could be used to the very last drop, and sufficed, when necessary, for several dozen estimations.

Human corpuscles were used, because they were found to be more easy to work with than those of the rabbit, which are smaller and more granular, and which also have a rather awkward tendency to stick together in clumps. The human leucocytes were quite active in the presence of rabbit's serum, and gave excellent results. There was a slight tendency to phagocytosis of red blood corpuscles, but not enough to cause any inconvenience.

Guinea-pig's serum on the other hand is a very unfavourable medium for human leucocytes, and in the experiments on these animals, guinea-pig's leucocytes were used. The preparation of a satisfactory suspension of guinea-pig's leucocytes at first presented some difficulty, because the blood begins to clot very quickly. Hence if blood from the ear was allowed to drop into citrate solution many small masses of fibrin had time to form, and to these the leucocytes adhered. The masses of clot and leucocytes, thus formed, were all dragged to the end of the film in the process of spreading, and bad preparations were obtained. The method finally adopted with success was to draw blood from the heart directly into some citrate solution. This can be done quite readily as follows. Into a sterile 1 c.c. syringe are drawn up ·75 c.c. of citrate solution. Then, the guinea-pig being held for the moment fairly and squarely on its back, on a holder, the needle of the syringe is plunged directly into the heart, entering just to the left of the sternum in an intercostal space. ·25 c.c. of blood are then aspirated from the left ventricle, and the mixed blood and citrate transferred to a small tube, containing a further quantity of citrate solution. This tube is well shaken at once, and the rest of the process of washing the corpuscles is proceeded with exactly as in the case of human corpuscles. Suspensions thus prepared gave excellent results.

The bacilli for the emulsion were removed by means of a platinum loop, from an agar slope culture, about 6 hours old, suspended in saline solution (·85 or 1 per cent.) and thoroughly mixed by the aid of a capillary pipette. This suspension was centrifuged for a minute or two, to precipitate clumps of bacilli, and the upper layers removed to a clean tube and mixed again, to insure uniformity. Emulsions were always prepared from young cultures (4 to 6 hours), as the bacilli are larger, stain better, and do not clump so readily.

The serum was obtained from the ear-vein of the rabbit to be tested. A capillary tube, drawn off to a fine point, forms the most convenient instrument with which to make a puncture. The blood was drawn into glass capsules containing about ¼ c.c.

IV. THE ACCURACY OF THE OPSONIC DETERMINATIONS.

The accuracy attainable in a series of opsonic measurements depends not only on the skill and patience of the observer and on the perfection of his methods, but on the species of the animal and microbe used. It is necessary therefore to enquire what degree of accuracy was actually attained in the experiments now before us.

1. *Accuracy of a single estimation.*

The accuracy of a single estimation is the first point to be considered. Having made one determination of the opsonic ratio between sera A and B, the question is how near this value is likely to be to the true value. Since the whole argument depends on the observation of *changes* in the opsonic indices of the experimental animals, it is of vital importance to be able to say, that the deviation of a single estimation from the true value will be *small in comparison with the changes which are to be observed.* In the absence of data for a complete mathematical statement, the following method of dealing with the problem may appear reasonable. In several series of observations the indices of all the pathological or inoculated rabbits were determined in duplicate, the counts, as also throughout the rest of these experiments, being made in ignorance of the source of every preparation. Of 210 such pairs of duplicate observations, only 2 show a divergence great enough to affect the form of the curve, of which each should define a point. Of the other 208 pairs, either member may be taken at will, or the curve may be drawn through the mean points, and those characters of the curves, from which deductions are to be drawn, remain entirely unaltered.

Reference to Figs. 29, 30, 31, 37 to 45, and 50, 51, 52 will render this point clear.

2. *The variation of the control.*

It was found that normal rabbits showed considerable differences in the opsonic power of their sera. These differences were more or less constant, so that a rabbit which had a low opsonic power on one day, would present the same peculiarity for many weeks. This property was not, however, invariable. The normal count, on which the indices of other sera were to be calculated, was therefore obtained by taking the mean of the counts of three or four normal rabbit sera. In the experiments done at the Lister Institute three normal rabbits were used, in those done in Cambridge four. In each case these standard normal rabbits were chosen, at the beginning of a series of experiments, from amongst a considerable number of normal rabbits. The opsonic powers of the sera of the whole batch were first determined, and the normals for future use were selected as giving a fair mean sample of the batch. Under these conditions it was found that a satisfactory control was established. That is to say, no rabbit, when compared with this control, ever showed accidental variations of anything like the magnitude of those obtained after inoculation or in disease; and on the other hand, experiments could be repeated, and gave constantly the same results. The only exception to this, was in the case of inoculations of diseased animals, where the variable conditions of disease made it extremely difficult to obtain an *exact* repetition of a previous experiment.

Towards the end of these experiments one of the control animals exhibited considerable fluctuations of the index, which roused the suspicion that it had become infected accidentally. Its index then rose and was maintained at a high level for several days, confirming this suspicion (Fig. 27). The animal was therefore sacrificed, and it was found to show the changes typical of a naturally acquired infection. There were nodules in the lymphoid tissue of the vermiform appendix, and of the expanded lower end of the ileum, two small nodules in the liver, and caseating areas in the portal and mesenteric glands.

3. *The variation of the emulsion.*

It has been pointed out by Houston (1907) and Wright (1908)[1] that in the case of some microbes, especially the Meningococcus and *B. coli,* very different results may be obtained when bloods are tested with different cultures all derived from the same stock. "For in association with the attenuation which these microbes undergo upon artificial media they gradually become less and less resistant to the phagocytic attack of normal blood. A comparatively low index is now obtained where before a very high index was obtained, the result being of course simply due to an increase in the denominator in the fraction:

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[1] Wright, *Practitioner*, May 1908, p. 586.

$$\frac{\text{phagocytic count of the patient's blood}}{\text{phagocytic count of the normal blood}}\text{.”}$$

This phenomenon is found to occur in connection with the *B. pseudotuberculosis*, as is shown in Figs. 1 to 4. For this experiment two cultures of the bacillus were used, (1) strain M, kept on artificial media, with frequent sub-culture, for seven weeks since isolation from its

Fig. 1. Rab. 10. Fig. 2. Rab. 6.

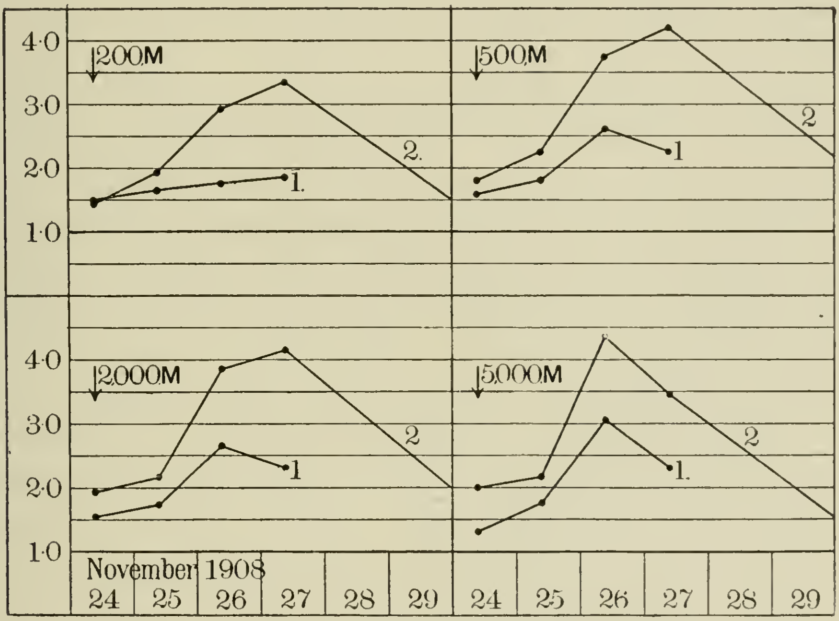

Fig. 3. Rab. 8. Fig. 4. Rab. 7.

Figs. 1 to 4. Effect of subcutaneous inoculations of vaccine, in rabbits recovering from a previous infection. Influence of attenuation of the culture used in making the opsonic determinations. Curve 1, in each figure, was obtained by using for the estimation of the index a stock which had undergone frequent subculture on agar for 7 weeks; Curve 2, in each figure, by using a stock of the same ancestry, but recently isolated from a rabbit.

Short arrows are used in all figures in this paper to mark the date of inoculations of killed cultures; long arrows denote inoculations of living cultures. The doses are given in millions of bacilli per kilogramme of body weight, or as fractions of a 24 hours' agar slope culture. For the relation between these two measures see Table II.

last passage through a guinea-pig; (2) the same strain which during these weeks had passed through two rabbits, recently isolated from the second. Four rabbits were inoculated with a vaccine made from culture 2. Before inoculation (Nov. 24th), culture 2 gave a higher index than culture 1 in three cases, and a slightly lower index in one case. After inoculation, the indices all rose, but those obtained with culture 2 were in every case higher than those obtained with culture 1. This experiment shows that attenuation of the culture, which is used for the emulsion, may have a great effect on the value of the index obtained. But the process of modification is gradual and progresses in one direction, and so does not interfere with the recognition of changes in the opsonic index induced by inoculations, which changes are more rapid, and have phases both of increase and decrease. This phenomenon of attenuation, however, prevents us from making any trustworthy comparison between the exact heights of the opsonic rises, observed in experiments carried out at different times, or with different strains of the bacillus.

It should be added that the phagocytic counts obtained with pathological sera, giving indices below normal, are increased, during the use of attenuated cultures, in a still greater proportion than the normal counts. Consequently while the normal counts approach those of highly immune sera, low counts approach more nearly to the normal, so that there results a levelling effect. A series of indices estimated with an attenuated culture preserve the order of their relative positions, but all are nearer the normal. It will be seen that this fact provides a further test for the detection of attenuation during a continued series of daily observations, wherever the number of animals under experiment contains individuals with both high and low indices. An accidental variation in the mean of the normal counts, would cause a corresponding alteration of all indices (both high and low) in the opposite direction to this variation. Attenuation of the culture, on the other hand, lowers the high indices and raises the low indices.

The process of attenuation has been described as gradual, but one cause of sudden attenuation has come under notice during this work. When a strain of the bacillus has been grown for some time on one brew of agar, transference to a fresh brew has several times produced a marked attenuation in the first culture on this new and slightly different medium.

4. *The kind and amount of selection exercised in presenting the results.*

In selecting the experiments, here presented, from the whole mass of experimental material collected during about 15 months' work, it was felt necessary to define the principle of what constitutes a fair method of selection, as opposed to an unfair method. If the methods of observation are liable to error, it is obviously possible to obtain, through error, a number of results which favour a preconceived opinion, whilst other results are contrary or doubtful. From such a total of data it is unfair to select those results alone, which favour the conclusion drawn. On the other hand, where all the data obtained favour one conclusion, confirming each other, it is fair to select for presentation a typical series of experiments. Following this principle, attention must here be drawn to certain observations which have been rejected, because they were of doubtful interpretation. The reader will then be able to judge whether the conclusions drawn from the published experiments are materially weakened by these doubtful results.

One curve was rejected because it contained one of the two pairs of widely divergent duplicates mentioned above, the rest of this curve was in harmony with the three given in Figs. 40, 41, and 42, which it confirms. The observations of one day (Feb. 13, 1909) were rejected because of the sudden attenuation of the culture used on this day, as shown by the fact that the indices obtained (7 high and 4 low) showed a marked tendency to approach the normal. Reference to Figs. 11, 12, 15, 16, 17, 46, 47, 48, 49, will show that the indices obtained on the 12th were distributed between 0·35 and 2·2. Those obtained on the 13th fell all between 0·64 and 1·78, ten out of eleven indices having come considerably nearer to the normal[1]. On the 14th a less altered culture of the same strain was used, and six out of seven indices showed again wider deviations from the normal, the seven being distributed between 0·26 and 2·5. It was clear, under these circumstances, that the measurements of the 13th had been made with a different scale to those of the preceding and following days, and they were omitted from the printed curves as obscuring their true form. In addition to the rejections explained above, a considerable number of experiments have been omitted as only serving to confirm points already amply demonstrated in the published figures.

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[1] These indices are shown on the charts by small circles.

V. The importance of opsonic immunity.

The general proposition that a state of well-being in the patient of a bacterial infection, is correlated with a raised opsonic index, is supported by a considerable mass of evidence obtained from observations upon man[1]. The following experiment, upon this subject, is given here, not as adding very much to the accumulated evidence of experience, but because it relates to the particular disease under consideration. I hope to be able shortly to carry out further experiments on somewhat similar lines.

Ten guinea-pigs were taken, whose opsonic indices had been estimated on two previous occasions, and found to differ widely from each other. It was thought likely that they would show corresponding differences in their powers of resisting an infection. Such wide differences, in the opsonic indices, are usually found, with regard to this bacillus, in a batch of presumably normal guinea-pigs, in which an autopsy reveals no sign of any previous infection. The disease is so fatal to guinea-pigs, that one can hardly suppose that a considerable percentage of adults have, at some time, acquired a relative immunity by way of the alimentary canal. One would be more inclined to regard these differences as natural or inborn, and comparable to the variations from the normal observed by Wells (1908) in healthy infants; but whichever view be correct matters little to the present argument. These ten guinea-pigs were inoculated on the same day, all with equal doses of the *B. pseudotuberculosis*, given intraperitoneally. The dose used was a small one, namely the one hundred thousandth part of a 24 hours' agar slope culture of the bacillus, isolated two days before from the heart's blood of a guinea-pig, dead of the disease. The animals were left untreated under the same conditions, and the course of the disease was observed. Determinations of the opsonic indices were made from time to time. Within ten days of the date of infection, seven of the guinea-pigs had died. The opsonic histories of all ten were then sorted into three groups, according to the period for which each guinea-pig had survived infection. A different opsonic curve was then seen to be characteristic of each of these groups, the opsonic curves in the same group showing a general agreement in form. A mean curve was therefore constructed for each group, and the three mean curves are presented on Fig. 5.

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[1] Macdonald (1906), Wright (1907, 1908), Inman (1908), Hektoen (1907), Banks (1907), Bassett Smith (1907).

It is seen that the three guinea-pigs which lived longest (group I.)[1] had shown high indices previous to infection, and also showed a capability to produce a relatively large increase in opsonin, in response to the stimulus of disease. The animals, on the other hand, which died earlier (groups II. and III.), were those with a past history of medium or low indices, and their indices remained low during the disease.

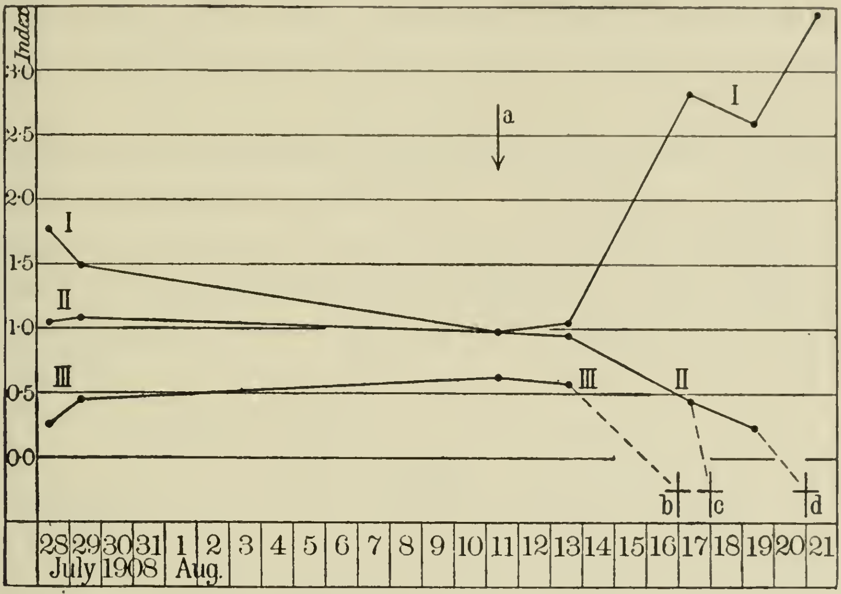

Fig. 5. Correlation between a good opsonic response, and an increased power of resisting infection.

Curve I. Mean opsonic curve for 3 guinea-pigs which survived the longest.

Curve II. Mean opsonic curve for 6 guinea-pigs which survived from 6 to 10 days.

Curve III. Opsonic index of 1 guinea-pig which only survived 5 days.

a. Date on which all the guinea-pigs were infected intraperitoneally, each with $\frac{1}{100000}$ of a culture.

b. One guinea-pig died.

c. Four died.

d. Two died.

VI. EFFECT OF INOCULATING NORMAL RABBITS WITH KILLED CULTURES OF *B. PSEUDOTUBERCULOSIS*; CONTRAST BETWEEN SUBCUTANEOUS AND INTRAPERITONEAL INOCULATION.

1. *Subcutaneous inoculation.*

The material for inoculation consisted of an emulsion of bacilli in ·85 or 1 per cent. sodium chloride solution. The bacilli were washed off the surface of a 24 hours' agar culture with the saline fluid, and, after enumeration of the bacilli against the red corpuscles of normal blood, by the method of Wright, the emulsion was heated to 60° C. for half an hour or an hour. Doses were estimated numerically, as so many millions of bacilli per kilogramme of body weight of the inoculated rabbit. For convenience this is denoted by the abreviation M. which is used, in citing doses, to mean *millions of bacilli per kilogramme of body weight.* Doses were usually made up to the volume of about 1 c.c. for subcutaneous inoculation. Some very big doses had, however, a larger volume.

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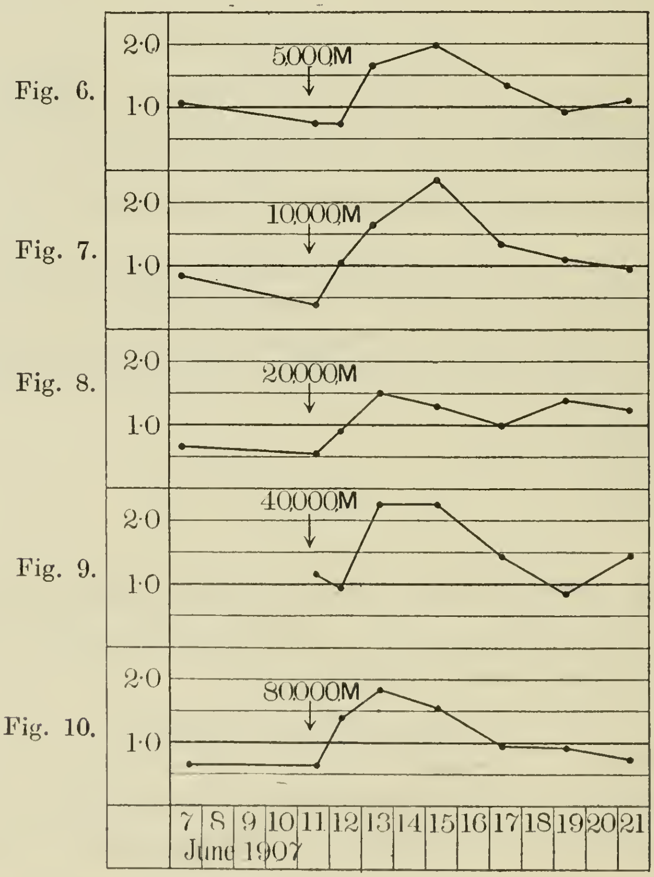
[1] Of these three guinea-pigs two died within a fortnight of infection, one recovered, and was killed by accident after 6 months. No traces of the infection were discovered at the autopsy.

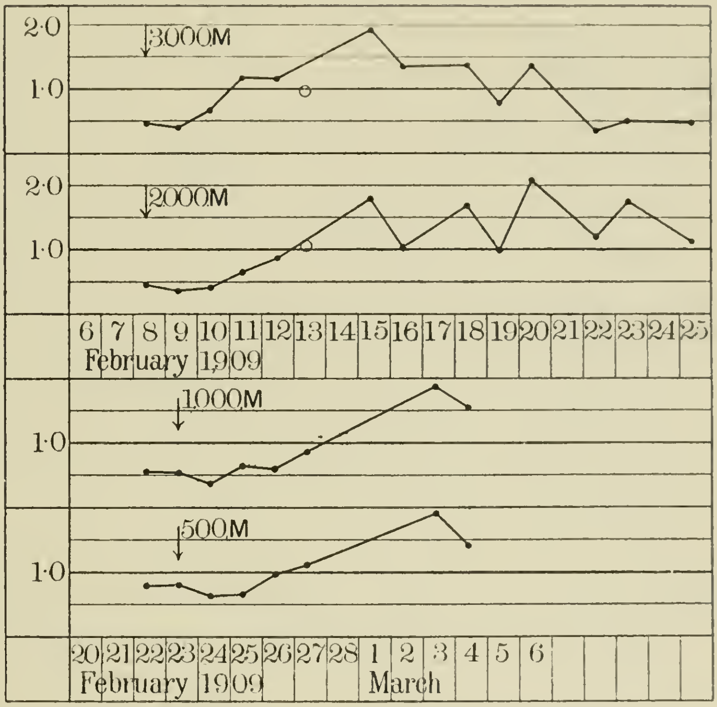
A series of normal rabbits received subcutaneous inoculations ranging from 160 M. to 80,000 M. Doses from 5000 M. up to the largest given, all produced the same response, as measured by the *rapidity and duration* of the rise in the opsonic index, which followed the inoculation (Figs. 6 to 10). Reference to these figures will show that the general



Figs. 6 to 10. Response of normal rabbits to subcutaneous inoculation of large doses of vaccine.

form of the curve is the same in all five cases, the differences between the curves being probably within the limits of error at this early period of the work. This form of response has been regarded as the typical form for subcutaneous inoculation, and the *minimum excitatory dose* (5000 M.), necessary to call forth this immediate reaction in a normal animal, is referred to in what follows as the M.E.D. (subcutaneous). Various doses, given in disease, or given intraperitoneally, are compared to it as to a standard. Doses smaller than 5000 M. produced a response which was delayed for some days, and did not as a rule reach so high a maximum (Figs. 11 to 14); delay was the most marked feature, characteristic of the response to small doses, in contrast to the immediate effect produced by the larger doses.

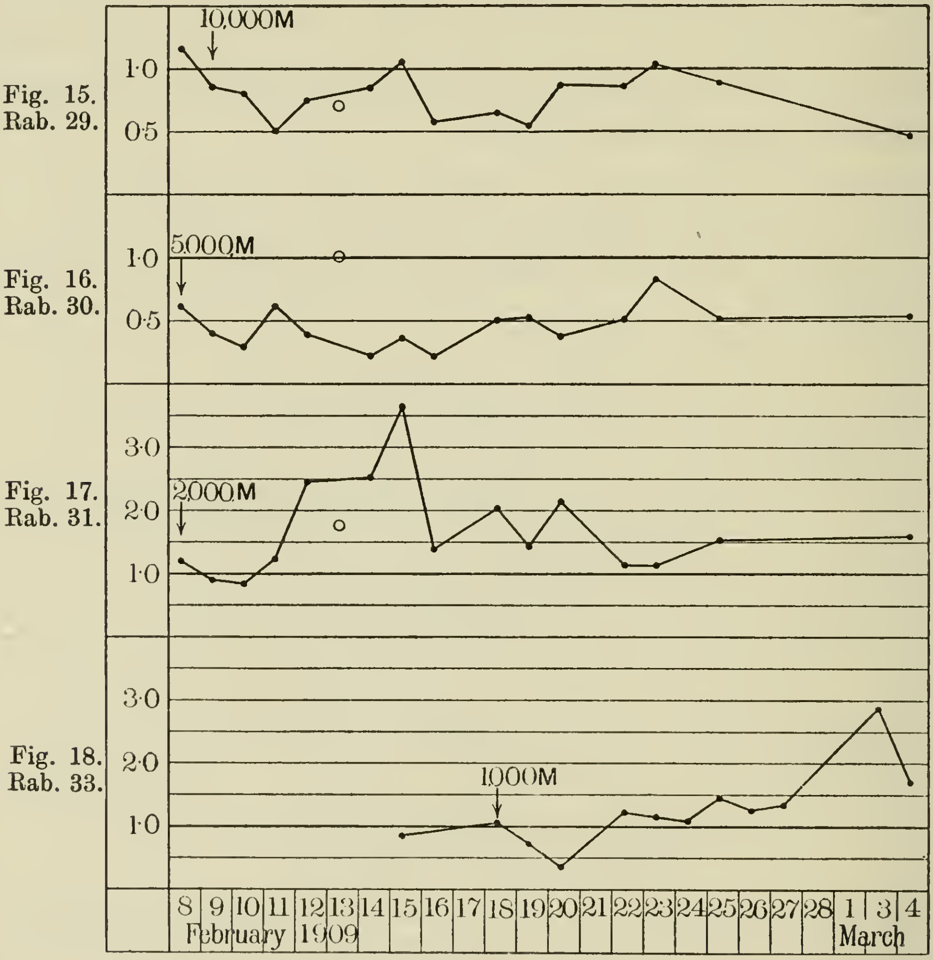
Fig. 11. Rab. 27.
Fig. 12. Rab. 28.
Fig. 13. Rab. 36.
Fig. 14. Rab. 37.



Figs. 11 to 14. Response of normal rabbits to subcutaneous inoculation of smaller doses of vaccine.

2. *Intraperitoneal inoculation of normal rabbits.*

When the vaccine is given intraperitoneally the response evoked is quite different from that obtained by subcutaneous inoculation. The most striking feature observed is a preliminary fall of the opsonic index (Figs. 15 to 18), a fall which is more marked and more prolonged the larger the dose used. The most favourable dose seems to be one of about 2000 M., for the rabbit which received this dose showed a very marked rise, following an initial fall of the index, which lasted for two or three days (Fig. 17). This contrast between the effects of subcutaneous and intraperitoneal inoculations, is a strong argument in favour of the local production of antibodies[1]. It appears, moreover, that the *B. pseudotuberculosis* is a microbe peculiarly well adapted for the exhibition of the differences of reaction, which are due to inoculation at different sites. This may be explained, in all probability, by supposing that the bacillus produces only a very limited amount of soluble toxic matter, so that the effects of injection of a vaccine are limited more strictly to the site of inoculation, than in the case of many other microorganisms. For it has been found to be true in most cases that subcutaneous injection of an antigen is followed



Figs. 15 to 18. Response of normal rabbits to intraperitoneal inoculation of vaccine.

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[1] Wassermann and Takaki (1898), P. Romer (1901), v. Dungern (1903), Wassermann and Citron (1905).

in the first instance by a decrease in the quantity of the corresponding antibody circulating in the body fluids. This was first shown by Brieger and Ehrlich (1892), who estimated the antitoxin content of the milk of a goat, which was inoculated with tetanus toxin. Salomonsen and Madsen (1897) confirmed these observations, by estimations carried out on the blood and milk of a mare, inoculated with diphtheria toxin. Wright (1901, 1903) showed that similar effects followed the inoculation of antityphoid vaccine; his observations referring to the bactericidal power of the blood serum. Wright (1907) quotes similar results again, with respect to the opsonic and agglutinating powers of the blood of men and animals, inoculated with various bacterial vaccines. Further, it has been shown that, in some cases, during this period of low antitropic power, the injected antigen may be detected in the circulating blood (von Dungern 1903). Under these conditions we cannot hope to observe the pure effect of a local inoculation, as it may be masked by the diffusion of the inoculated material through the system. Conversely, in a case where inoculations in different situations produce widely different effects, we may surmise that these effects are chiefly due to a localised action. In the case of the *B. pseudotuberculosis*, its low toxicity provides another argument for the very small amount of its soluble poisonous products; large numbers of living bacilli have to be present in order to kill an infected rabbit, and extraordinarily large doses of vaccine have to be used in order to produce any effect on a normal rabbit. This is clear when one remembers that the prophylactic dose of *B. typhosus* for a man contains about a thousand million bacilli, and very few therapeutic doses as large as this have been used in respect to any of the bacterial infections of man, which have been inoculated with success. While therefore a thousand million of the bacilli, with which we are familiar, usually constitute an exceptionally large dose for a man, a normal rabbit weighing 2 kilogrammes must receive subcutaneously ten times this number of *B. pseudotuberculosis* before the typical response is produced. Parallel with this low degree of activity exists a considerable difficulty in producing a low opsonic index even in a diseased animal by subcutaneous inoculation with this bacillus (Part VIII. p. 203), which seems, therefore, to form very little nocuous material in a soluble form.

From the above it will be seen, that the effect of subcutaneous inoculation of pseudotubercle vaccine differs both from that of intraperitoneal inoculation of the same vaccine, and from that of subcutaneous inoculations of soluble toxins, or of vaccines which contain much soluble antigen. The most probable reason for this is, that we are here studying the effect of a purely local response of the subcutaneous tissues at the site of inoculation. On the other hand, when the vaccine is given intraperitoneally, the very rapid interchange of lymph between the peritoneal cavity and the general circulation, gives every opportunity for the absorption of the circulating opsonin, even if the vaccine material is not itself washed into the blood stream. If this be granted it will follow that the subcutaneous tissues are specially adapted to perform a protective *rôle*, against bacterial invasion. This *rôle* they fulfil by reason of their peculiar ability to react rapidly to a sufficient stimulus, and to dispose, in some way, of an excess of bacterial matter, without allowing the general stock of circulating opsonin to be diverted in order to combine with this surplus. It remains for future investigations to determine how far this speciality is confined to the subcutaneous tissues. The comparison here is only between these and the peritoneal system, by which term is denoted the peritoneal cavity and the extensive lymphatic areas, which are in easy connection with it. It is possible in view of the success which has attended the method of immunisation by way of the gut, that the mucous or submucous tissues may possess powers similar to those of the subcutaneous tissues. The experiments of Leishman (1908), however, with typhoid vaccine weigh against this surmise. It should also be pointed out that, when immunity has been established, other tissues acquire this property of rapid response, as will be shown in a later part of this paper[1].

VII. INFECTION WITH LIVING CULTURES, INJECTED INTO THE PERITONEUM OR INTO THE BLOOD STREAM.

If we introduce a living emulsion into the peritoneal cavity the series of events which follows this infection, is again different from either of the cases treated in the preceding part. If the rabbit is to survive for more than a day or two, the dose must not exceed $\frac{1}{5}$ of a 24 hours' agar slope culture, which quantity contains about 6,000,000,000 bacilli, of which nearly all are living (see Table II).

The injection of such a dose as $\frac{1}{5}$ of a culture is followed by a considerable fall of the opsonic index, which is maintained for several days (Figs. 19, 20, 21). It has been shown in Part VI. that such a fall may be produced by intraperitoneal injection of killed bacilli (Figs. 15, 16, 17, 18), but cannot be produced by subcutaneous inoculation. The living bacilli are, therefore, just as well able to abstract opsonin from the circulating fluids, as those killed by heat. On the other hand, even with their power of multiplying and spreading into the substance of the abdominal organs, they do not form any more efficient stimulus to the defensive mechanism of the animal. The production of increased quantities of opsonin is delayed just as much in the case of the living inoculation, as it was in the case of the dead vaccine. When a smaller quantity of living microbes are used for the inoculation, that is a quantity less than the minimal efficient intraperitoneal dose of bacilli killed by heat, there is an additional period of delay, while the infecting microbes have time to reach the minimal number efficient as a stimulus. This is illustrated by the experiment shown on Figs. 32 to 36 where the dose inoculated was $\frac{1}{100}$ of a culture, or about equivalent to an inoculation of 150 M.; also by Fig. 19, which represents the mean of 9 curves, obtained in experiments where the infecting doses ranged from $\frac{1}{100}$ to $\frac{1}{4}$ of a culture. From these observations it would appear that, though the bacilli have early invaded and occupied in force the omentum, liver, spleen, and abdominal lymphatic glands, and though they are even circulating in the blood stream, yet they have not, at this time, gained access to any tissue which has the power (presented by the subcutaneous tissues) of a prompt and vigorous immunising response.

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[1] Compare also Wassermann (1905), *Deutsch. med. Wochenschr.* p. 1101.

TABLE II.

Number of culture tubes	Average number of microbes per tube, enumerated against red blood corpuscles	Number of living microbes by culture
4	28,000,000,000	—
1	31,000,000,000	26,000,000,000
1	31,000,000,000	31,000,000,000

After an inert period of shorter or longer duration, during which the opsonic index remains low, a reaction at length sets in, and the index rises to a high level which is only maintained, however, for a few days (Fig. 22). After this the opsonic index shows a tendency to fall gradually, though further autoinoculations occur from time to time, inducing reactions which often surpass that first produced, in intensity. An example of this was seen in the case of rabbits 19, 20, 21 and 22, whose indices rose during the first reaction to little over 2 (Figs. 32 to 36), whilst a fortnight later they were found to have attained an average value of about four (Figs. 39 to 42; Jan. 1 and 2).

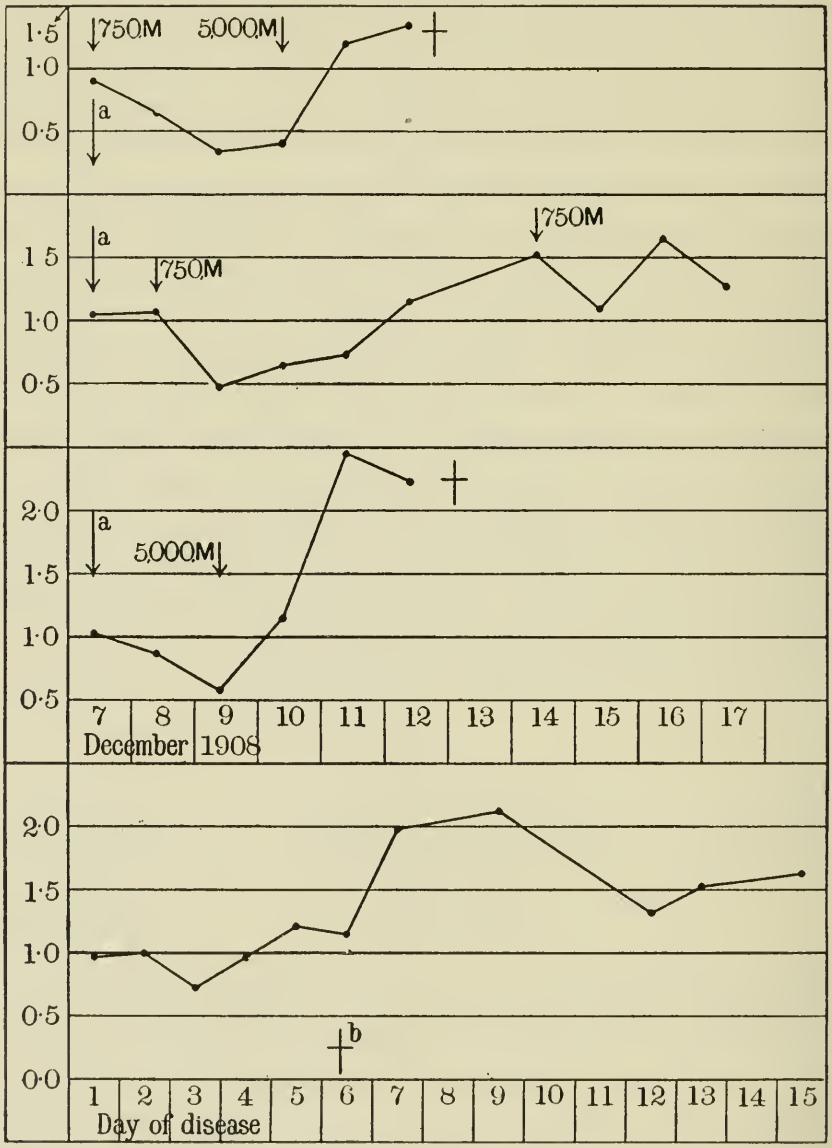
Of the rabbits infected with moderate doses (less than $\frac{1}{5}$ of a culture), four died before the first reaction set in (Figs. 22 and 36), three on the sixth day, and one on the tenth day of the disease; whilst all those which survived the inert period, and produced an immunising response, as shown by the opsonic index, went on to ultimate recovery; none died. Of those which received $\frac{1}{5}$ or $\frac{1}{4}$ of a culture, on the other

Fig. 19. Rab. 24.

Fig. 20. Rab. 25.

Fig. 21. Rab. 26.

Fig. 22.



Figs. 19 to 22. Response of normal rabbits to intraperitoneal inoculation of living cultures.

Figs. 19 to 21 show also the effect of various subsequent subcutaneous inoculations.

Fig. 22. Composite curve of opsonic indices of 9 rabbits which were infected with living bacilli on day 1.

+ signifies the death of the animal.

a. Three out of the 9 rabbits died.

hand, three out of four died (Figs. 19, 20, 21), in spite of an immunising response, elicited in two cases by the subcutaneous injection of vaccine and in the third case supervening in the ordinary course of the disease

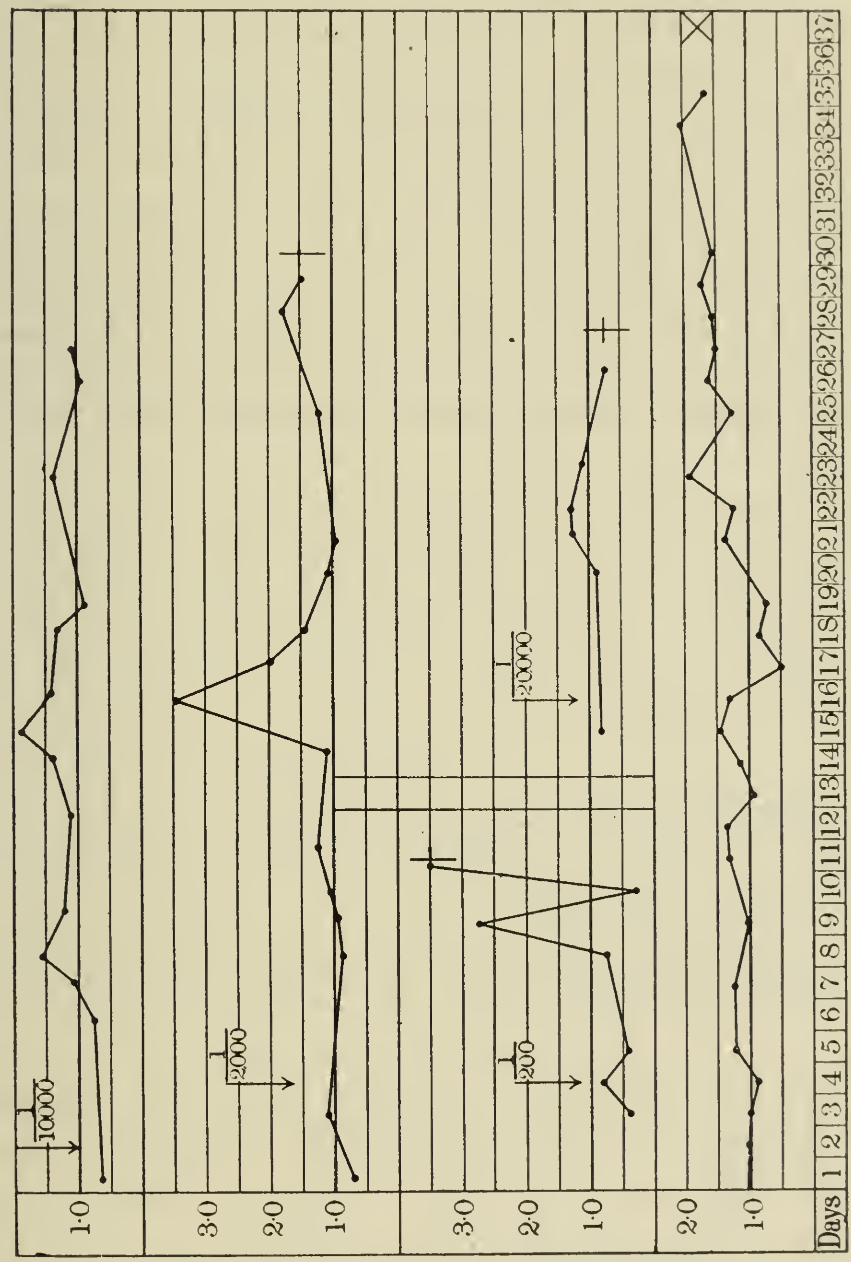


Fig. 23. Rab. 25. L.

Fig. 24. Rab. 23. L.

Fig. 25. Rab. 16. L.

Fig. 26. Rab. 24. L.

Fig. 27. Rab. 15. L.

Figs. 23 to 26. Response of normal rabbits to intravenous inoculation of living cultures.

Fig. 27. Variations occurring in the opsonic index of a rabbit during the development of a naturally acquired infection. The exact date of the infection is not known.

× The animal was killed, and the diagnosis confirmed.

(rabbit 25, Fig. 20, died on the 26th day of the disease). It was, of course, only to be expected that a massive infection would be able to overcome the protective mechanism of the animal, even when excited to its utmost activity.

Intravenous inoculation of living cultures produces a more fatal disease than intraperitoneal inoculation. No rabbit survived infection by way of the blood stream with more than $\frac{1}{10000}$ of a culture. Some of the rabbits which received doses between $\frac{1}{20000}$ and $\frac{1}{200}$ of a culture lived long enough to produce an immunising response. In these the inert period was at least as long as that following infection by way of the peritoneum, but it was not accompanied by any marked decrease in the quantity of circulating opsonin (Figs. 26 to 29). The bacilli therefore do not multiply in the blood stream to any great extent (as may be also shown by blood-cultures), but they become lodged in various tissues throughout the body, and are shut off from the circulation, within the necrotic foci or abscesses thus formed, so that the amount of opsonin abstracted from the circulating blood remains comparatively insignificant. The immunising reaction, when it appears, is markedly fitful and ill sustained.

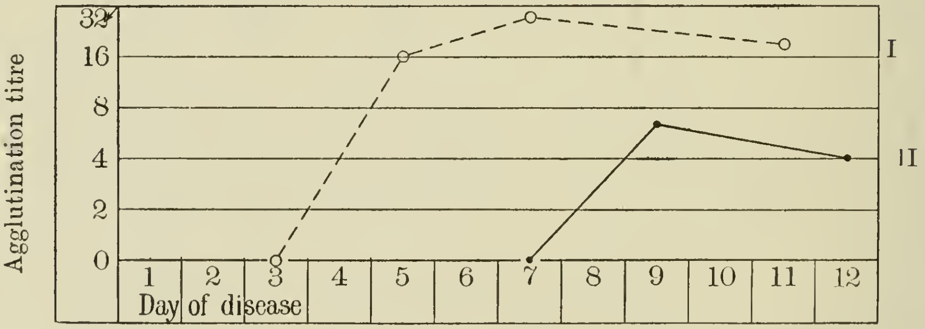


Fig. 28. Interrupted line; development of agglutinin in five rabbits which received subcutaneous inoculations of vaccine on day 1.

Continuous line; development of agglutinin in three rabbits which received intraperitoneal inoculations of living cultures on day 1. The figures at the left-hand side denote the dilutions of the serum, in which a distinct agglutinating reaction was obtained.

Fig. 27 shows the changes in the opsonic index, which occurred during the development of an infection, acquired accidentally by way of the alimentary canal.

Agglutination experiments.

The absence of any immunising reaction during the first period of the disease, which has been shown above by reference to opsonic observations on a series of 23 rabbits, may be equally easily demonstrated by agglutination experiments. Three infected rabbits, on which these experiments were made, only produced agglutinin after the lapse of eight days from the day of infection (Fig. 28; Curve II.). On the other hand, subcutaneous inoculation of five different normal rabbits, with various doses of killed bacilli, led to the discovery of a relatively high agglutination titre, in every case, after only four days from inoculation (Fig. 28; Curve I.).

VIII. THE RESPONSE ELICITED BY INOCULATION OF STERILISED VACCINE, IN RABBITS PREVIOUSLY INFECTED WITH THE DISEASE.

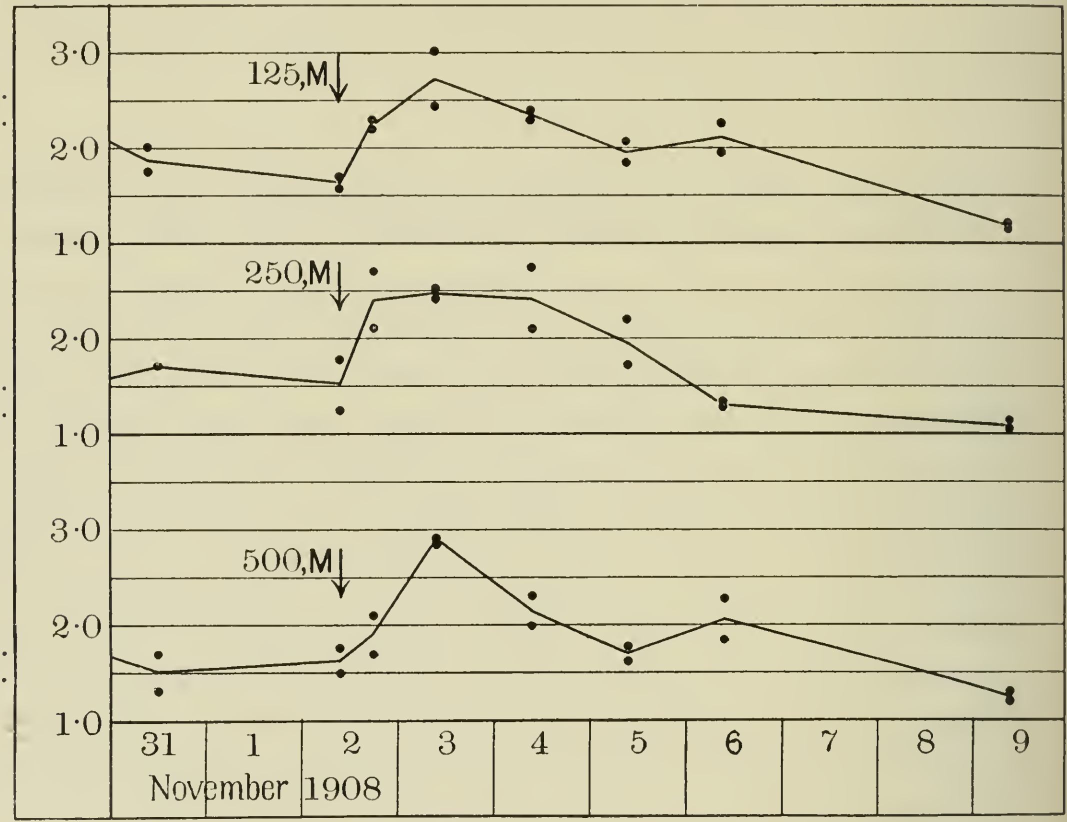
During the first stage of the disease caused by inoculating a living culture into the peritoneum, the experimental animal still reacts to a subcutaneous inoculation of vaccine in the same manner as a normal rabbit does. This fact enables us to state with more certainty the cause of the decrease in the opsonic content of the blood, which is observed during this period. This decrease was attributed solely to the abstraction of opsonin from the circulating fluids by the infecting bacilli. But there were two further possibilities, namely that the production of opsonin had been inhibited, and the supply thus cut off at the source, or, on the other hand, there might be an increased production of opsonin, which was hidden from observation because masked by the rapid absorption of this from the blood, by the bacilli. These possibilities are however disposed of by the fact mentioned above, which was established by the following experiment.

The three rabbits, 24, 25, 26, were inoculated, each with $\frac{1}{5}$ of a 12 hours' agar slope culture, on the same day. After 48 hours, rabbit 26 was obviously weaker than the other two, and appeared likely to die within the next 24 hours. It seemed that, if the presence of a severe infection can ever inhibit or mask the production of opsonins, this rabbit was in a condition to exhibit the phenomenon. It was therefore given a subcutaneous injection of 5000 M.—the M.E.D. for a normal animal. During the next two days the opsonic index rose to over 2·4 (Fig. 21). On the next day, rabbit 24, being at the time, as far as one could judge, *moribund*, received a similar subcutaneous inoculation. It lived long enough to show a rise of the opsonic index up to 1·34 (Fig. 19). It is clear that an appropriate stimulus, even in this stage of the disease, can excite the production of opsonin in increased quantity; an increase which the presence of a very grave infection is unable either to inhibit or to mask. The lowered index, which marks the beginning of this intraperitoneal infection, is therefore due only to the absorption of opsonin by the bacilli introduced; it is accompanied neither by inhibition, nor by any efficient stimulation of the immunising mechanism.

Fig. 29.
Rab. 8.

Fig. 30.
Rab. 10.

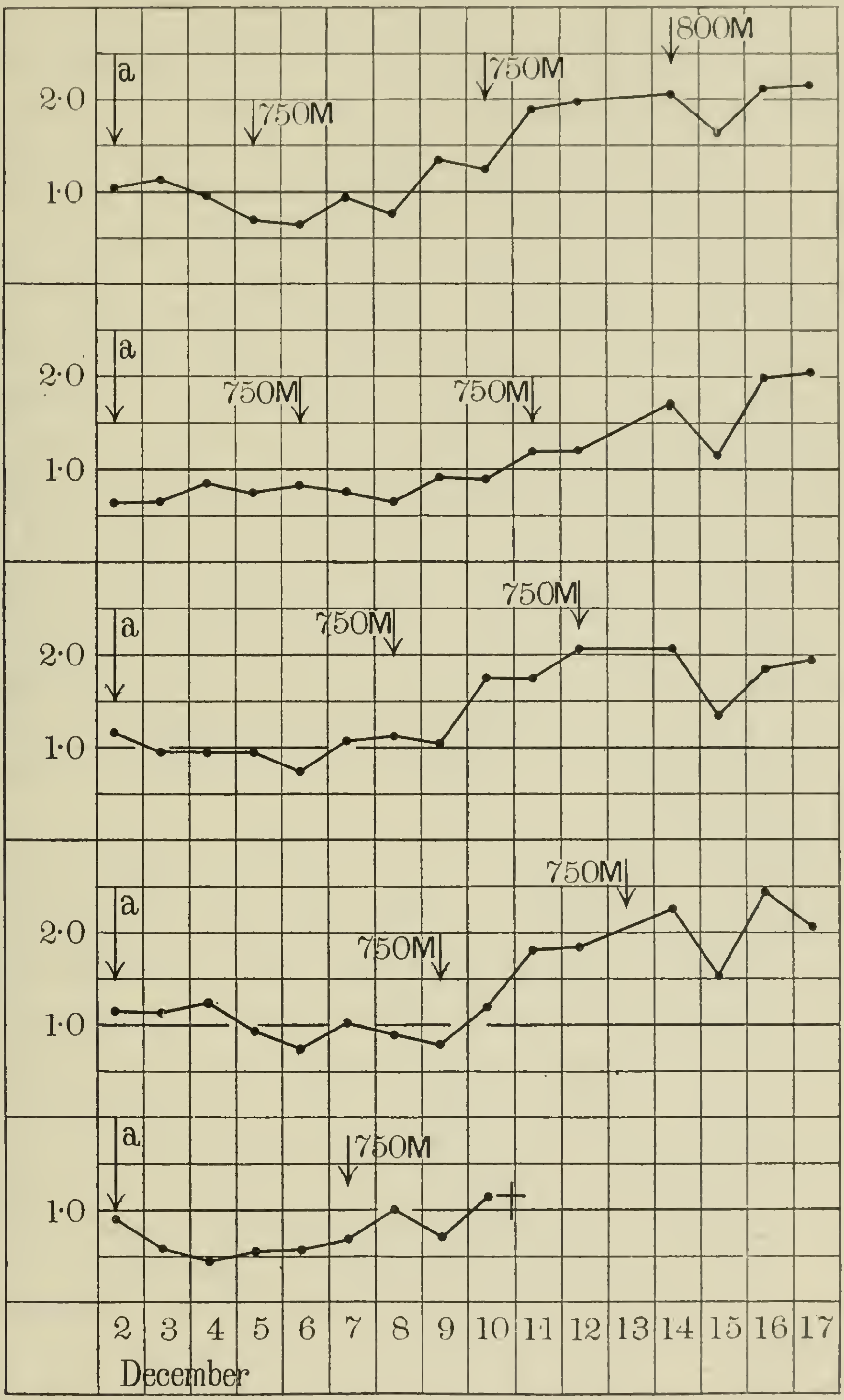
Fig. 31.
Rab. 7.



Figs. 29 to 31. Response of previously infected rabbits to small doses of vaccine, given subcutaneously.

After a certain degree of immunity has been attained in the course of the disease, the reactions of the animal towards injections of vaccine are entirely altered. In the first place it becomes possible to elicit a response with a dose one hundredfold less than the M.E.D., and secondly it becomes possible to cause a temporary lowering of the opsonic index, by subcutaneous inoculation of suitable doses of vaccine. The tissues of the (relatively) immune animal have acquired a new property, by virtue of which they react with increased vigour to the presence of microbes of the species which has induced the immunity. This tissue-immunity, which outlasts the presence of an increased quantity of antibodies in the body fluids, has been emphasised by Wassermann and Citron (1905).

The response to small doses was first obtained in three rabbits (Figs. 29, 30, 31), one of which received the inoculation on the 15th day of the disease (rabbit 10, Fig. 30). An experiment was therefore



Figs. 32 to 36. Opsonic charts of rabbits which were infected at (*a*) with doses of $\frac{1}{100}$ of a culture. Subsequent inoculations of moderate doses of vaccine, subcute, produced no immediate response.

instituted to determine when this property of sensibility to small doses first appeared. It was obvious that if test inoculations were made, the opsonic indices of the inoculated animals should give an indication of the effect, or absence of effect, of inoculation, within 24 hours.

For this experiment 5 rabbits were infected intraperitoneally on the same day with equal doses ($\frac{1}{100}$ of a culture) of the *B. pseudotuberculosis.* While their opsonic indices were estimated daily, they were given test doses of vaccine at intervals. The dose of vaccine chosen was 750 M. that is, 15 times the minimal effective dose for a sensitive immune rabbit, and rather less than $\frac{1}{6}$ of the M.E.D. (Figs. 32 to 36). Reference to the figures will show that certainly none of the inoculations before the eighth day were effective. Rabbit 22 (Fig. 35) gave a rise of index following immediately on an inoculation on the eighth day, but, at this time, one expected a rise in the ordinary course of the disease, so that it remains uncertain whether this dose had any effect or no. Similarly inoculations on the 9th and 12th days (rabbits 19 and 22; Figs. 32 and 35) had a doubtful effect, while doses on the 10th and 11th days (rabbits 20 and 21; Figs. 33 and 34) had no effect. In rabbit 10, however, as mentioned above, a dose of 250 M. given on the 15th day of the disease, had produced an undoubted response (Fig. 30). This rabbit had received a larger infecting dose, namely $\frac{1}{20}$ of a culture. The conclusion is, that the condition of increased sensibility to inoculation is only established some days later in the course of the disease than a high opsonic index.

The response of a diseased and sensitive animal to a small dose may have one of two forms. Either there follows immediately on the inoculation a very rapid rise of the opsonic index, which is already well begun within the first six hours, and which reaches its maximum in about 24 hours (Figs. 29, 30, 31), or there is a gradual rise, which begins slowly and goes on with increasing rapidity to a maximum after 48 to 72 hours. Such a response as this last has only been seen (in these experiments) in animals which had already received several doses of vaccine (Figs. 1, 2, 3, 4, 37, 38). The first form is that more familiar to those who have treated, by inoculation, various bacterial infections in man. Passing on to examine the effect of larger doses, it is found that the response elicited is irregular, and shows periods of decrease as well as of increase in the opsonic index (Figs. 39 to 45). The response in disease, therefore, differs from that of a normal rabbit, in the fact that a "negative phase" can be produced, which may or may not be preceded by a temporary rise of short duration, whereas in normal rabbits, subcutaneous injections of pseudotubercle vaccine are not followed by any negative phase (Figs. 6 to 14). The dose required to produce this effect in a diseased rabbit varies in different rabbits and in different stages of the disease, and is also influenced by the height of the opsonic index at the time of inoculation, and by previous inoculations. Thus doses of 500 M. (Fig. 45) and 410 M. (Fig. 42) were both sufficient to cause a temporary fall of the index, in rabbits not previously inoculated

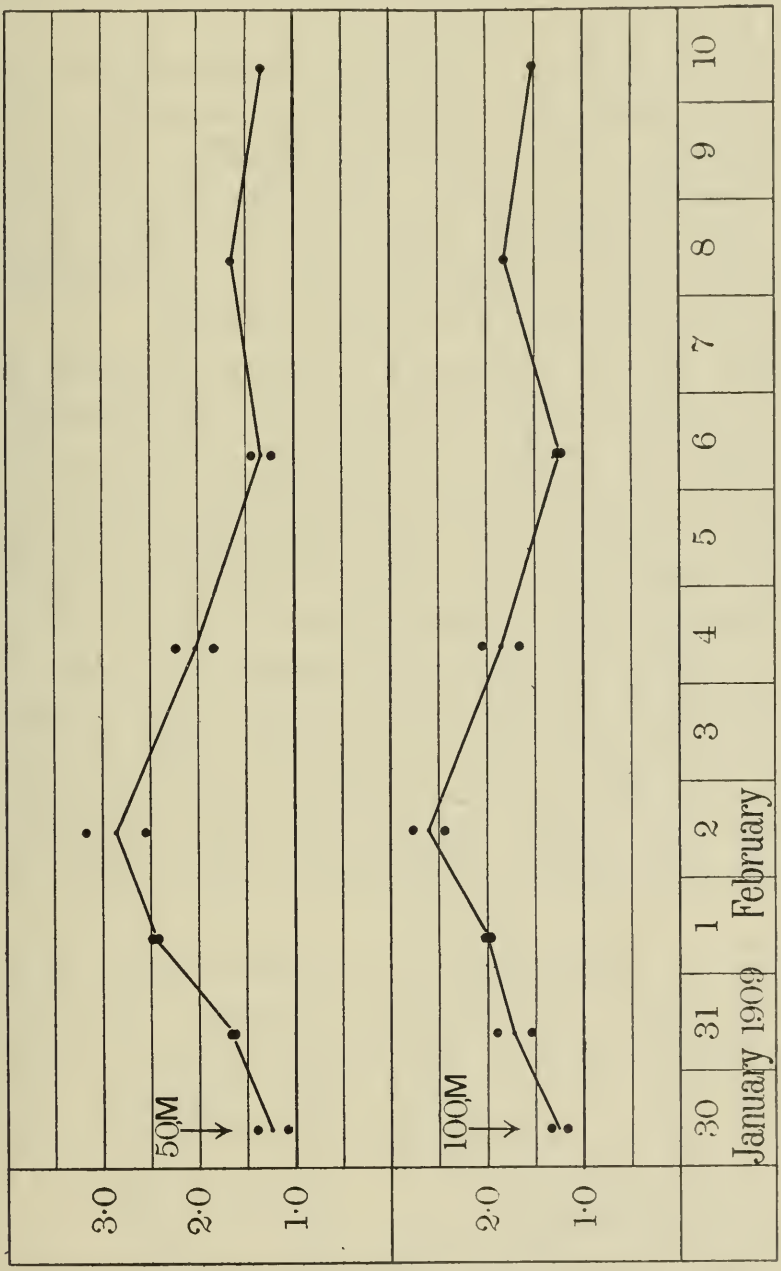


Fig. 37. Rab. 22.

Fig. 38. Rab. 20.

Figs. 37 and 38. Response of rabbits, recovering from a previous infection, to small subcutaneous doses of vaccine.

with an effective dose, whereas the inoculations of Nov. 24th (Figs. 1 to 4), ranging from 200 M. to 5000 M., all alike acted as small doses, that is produced a pure rise of the index in rabbits which had each been inoculated on several previous occasions. It may be pointed out that Figs. 32 and 45 refer to one rabbit, the interval elapsing between the two inoculations being five weeks, during which time 2 intervening inoculations were given. Similarly Figs. 30 and 46 refer to one rabbit; there was the same time interval, of five weeks, and 3 intervening inoculations.

The inoculations shown on Figs. 39 to 42, illustrate the ease with which an already high index is lowered by inoculation, of even a relatively small dose. The four rabbits used for this experiment all had indices before inoculation of about 4. The doses given on this occasion ranged from 410 M. to 50,000 M. and the response evoked was the same in every case, the most marked feature being a very rapid fall of the index during the first 24 hours succeeding inoculation.

With regard to this experiment it should be stated that the persistence of a lowered index was partly due to the alteration of the strain from which the emulsion was prepared each day. Thus the culture used on Jan. 1st was only the third subculture since isolation of the bacillus from a rabbit's blood. But as subcultures were made once, and sometimes twice a day, that used on Jan. 6th was considerably attenuated. This attenuation being suspected on Jan. 7th, a return was made to the second subculture since isolation, and a slope was sown from this. The indices obtained, with an emulsion of this culture, were all a good deal higher than those for Jan. 6th. There is, however, no doubt that the first effect of the inoculations of Jan. 2nd, was a very pronounced fall of the opsonic index of each rabbit; and this fall was produced in one case (rabbit 22, Fig. 42) by a dose less than one tenth the M.E.D.

To sum up the foregoing section: an animal which has a raised sensibility with regard to inoculation of vaccine, reacts differently according to whether small or large doses are given. Small doses produce a pure rise in the opsonic index, large doses produce a temporary fall; but the dividing line between what constitutes a "large dose," and what a "small dose," varies considerably, and depends on the condition of the animal.

With regard to the response of a diseased animal to inoculation, the question arises whether the dose administered may not suffice to induce an inflammatory reaction at the foci of disease, just as tuberculin or

Fig. 40. Rab. 20. Fig. 42. Rab. 22.

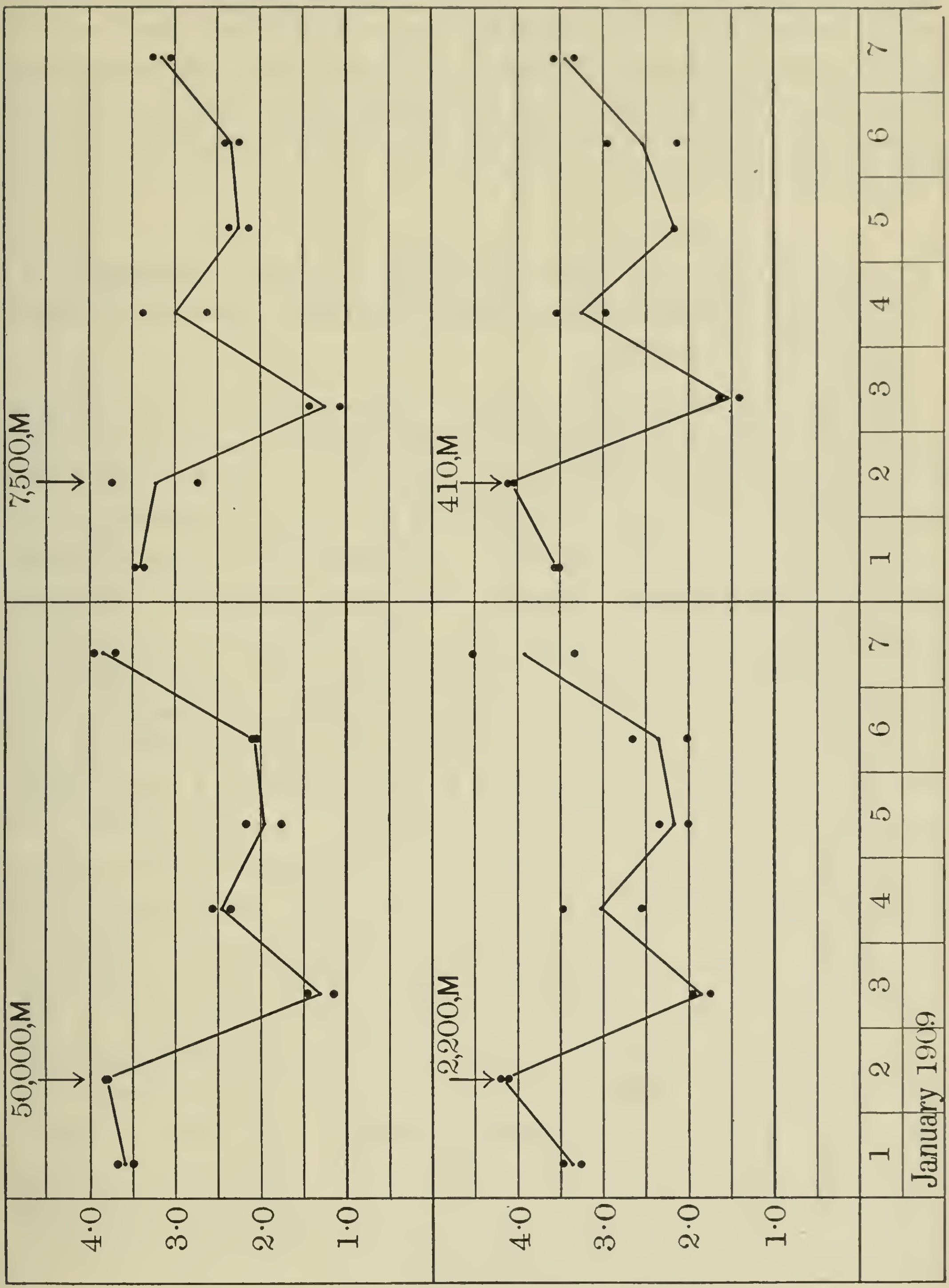


Fig. 39. Rab. 19. Fig. 41. Rab. 21.

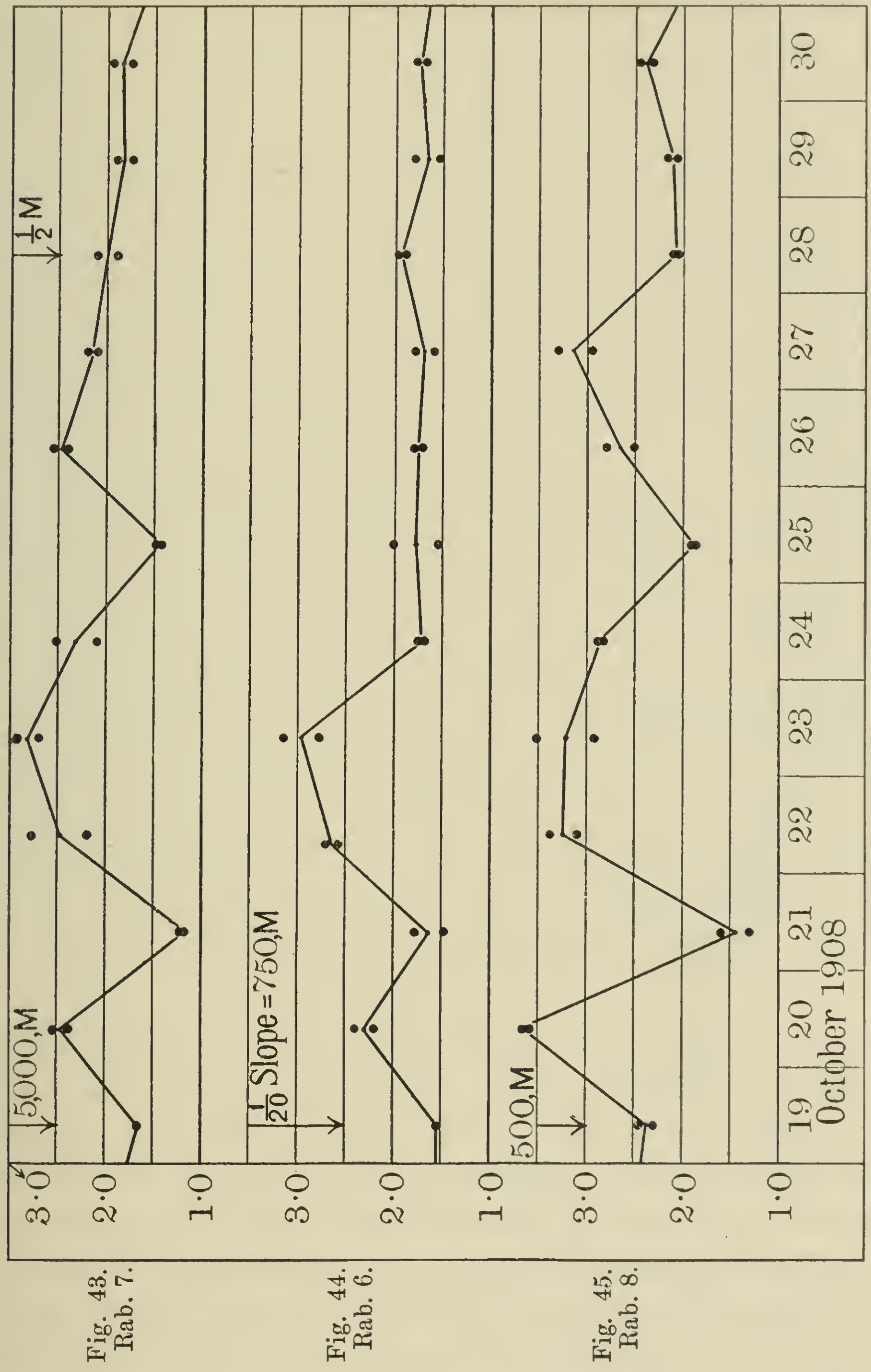
Figs. 39 to 42. Response of rabbits, previously infected, to large and moderate subcutaneous inoculations of vaccine. The index, which was very high in each case before inoculation, falls immediately.

mallein may, when used in veterinary practice as a test for tuberculous disease or glanders. Such a reaction, by increasing the circulation, would tend to carry the bacterial products more widely afield, and in larger quantity, and thus a secondary autoinoculation could conceivably follow as a consequence of an inoculation from without. For the present, however, we want sufficient data for the elucidation of this complex question.

IX. LIVING CULTURES AND STERILISED VACCINES CONSIDERED AS STIMULUS-MATERIAL, AND THEIR EFFICIENCY, FROM THIS POINT OF VIEW, COMPARED.

The question whether the antigen, which excites the production of opsonin, is a metabolic product of the growth of the bacillus, or, on the contrary, a product of its disintegration, is of no little interest from the point of view of vaccine therapy. If the first alternative were true, the inoculation of a living culture would provide not only a maximum quantity of ready-formed antigen, but also a source of continuous supply of fresh antigen. But if the second alternative be true, then it is possible that the process of sterilising the vaccine may increase its immediate value as stimulus-material, by breaking up the bacilli, and rendering them more readily available to the tissue cells. In this manner one could imagine a killed culture providing a more intense immediate stimulus, than a similar quantity of a living culture. To test this it was necessary to compare the immediate stimulus-value of bacilli, living, and killed by heat. In making this comparison one can only judge by the immediate results, because the later effects of inoculation would be influenced by the multiplication of the living bacilli within the body. Hence no conclusions can be drawn from the response following intraperitoneal injection, of killed and living cultures, into normal animals. In both cases the response is delayed, and multiplication of the living bacilli has had time to occur before it sets in. The effect of inoculating living bacilli into the subcutaneous tissues might be profoundly influenced by the gross changes accompanying abscess formation, so that this method of comparison between the living and dead would give untrustworthy results.

The immune animal, however, can react immediately to intraperitoneal inoculations of either living or dead cultures, so that a fair comparison could be made of their stimulus-value, by making injections into the peritoneal cavities of rabbits which had recovered from a previous infection, and were consequently in a condition of immunity. A suitable quantity of living bacilli, given in this manner, was found to excite a response similar to that called forth by the injection of killed vaccine, as will be seen by comparing Fig. 44 with Figs. 43 and 45. This form of response was obtained with widely different doses of vaccine (500 M. and 5000 M.), so that the experiment did not give exact information as to what was the vaccine-equivalent of the quantity



Figs. 43 to 45. Intraperitoneal inoculation of living bacilli (Fig. 44) compared with subcutaneous inoculation of vaccine (Figs. 43 and 45). The rabbits on which these inoculations were made were recovering from a previous intraperitoneal infection with living cultures.

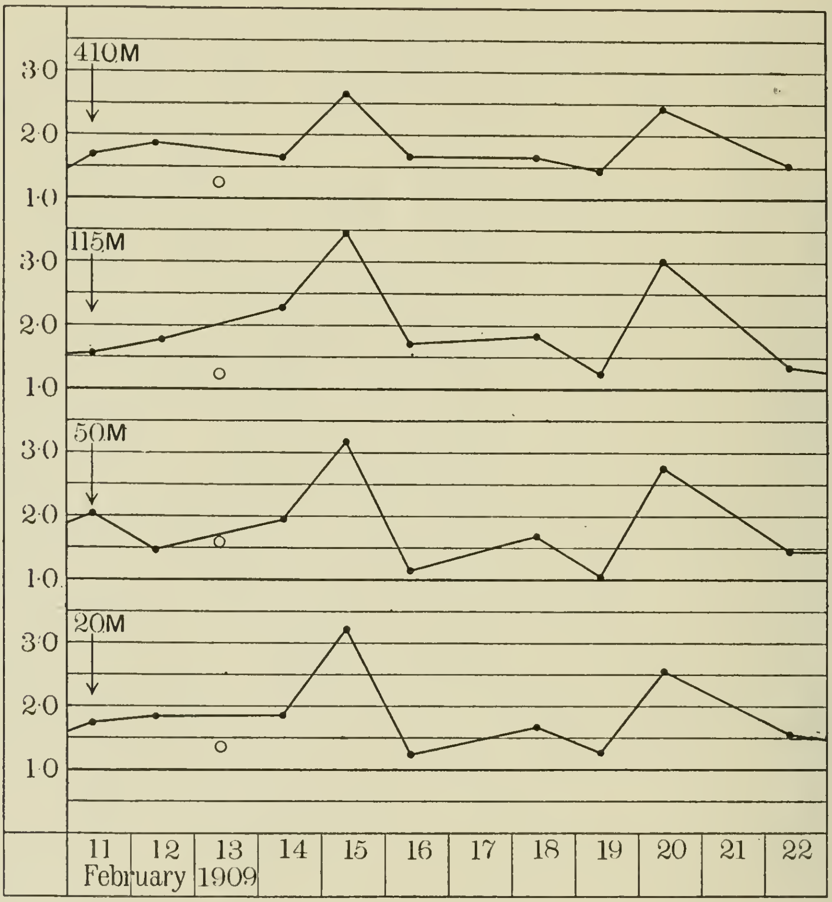
of living culture used ($\frac{1}{20}$ of a 24 hours' agar slope culture = 750 M.). A second experiment, made with smaller doses, gave more information. Four rabbits received respectively 20 M., 50 M., 115 M. and 410 M. (living culture); none of these rabbits gave an immediate response (Figs. 46 to 49), their indices remained steady for 3 days after inoculation, and only rose on the fourth day. These doses of living bacilli were, therefore, too small to excite at once an immunising response.

Fig. 46.
Rab. 19.

Fig. 47.
Rab. 20.

Fig. 48.
Rab. 21.

Fig. 49.
Rab. 22.



Figs. 46 to 49. Response of rabbits, recovering from a previous infection, to intraperitoneal inoculations of living cultures.

In contrast to this series, three rabbits were given respectively 50 M., 100 M. and 200 M. sterilised vaccine. They all gave a response, which began with a slight rise of the opsonic index on the first day, and reached its maximum on the third day after inoculation (Figs. 50 to 52). This shows that a dose of vaccine as small as 50 M. contains more immediately available antigen, than does the comparatively large dose (410 M.) of living bacilli. The living bacilli on the other hand, by the decay of successive generations, keep up a constant supply of antigen, which ultimately produces a corresponding reaction on the part of the animal. It will be observed that this reaction followed the smallest dose, as well as the largest dose given.

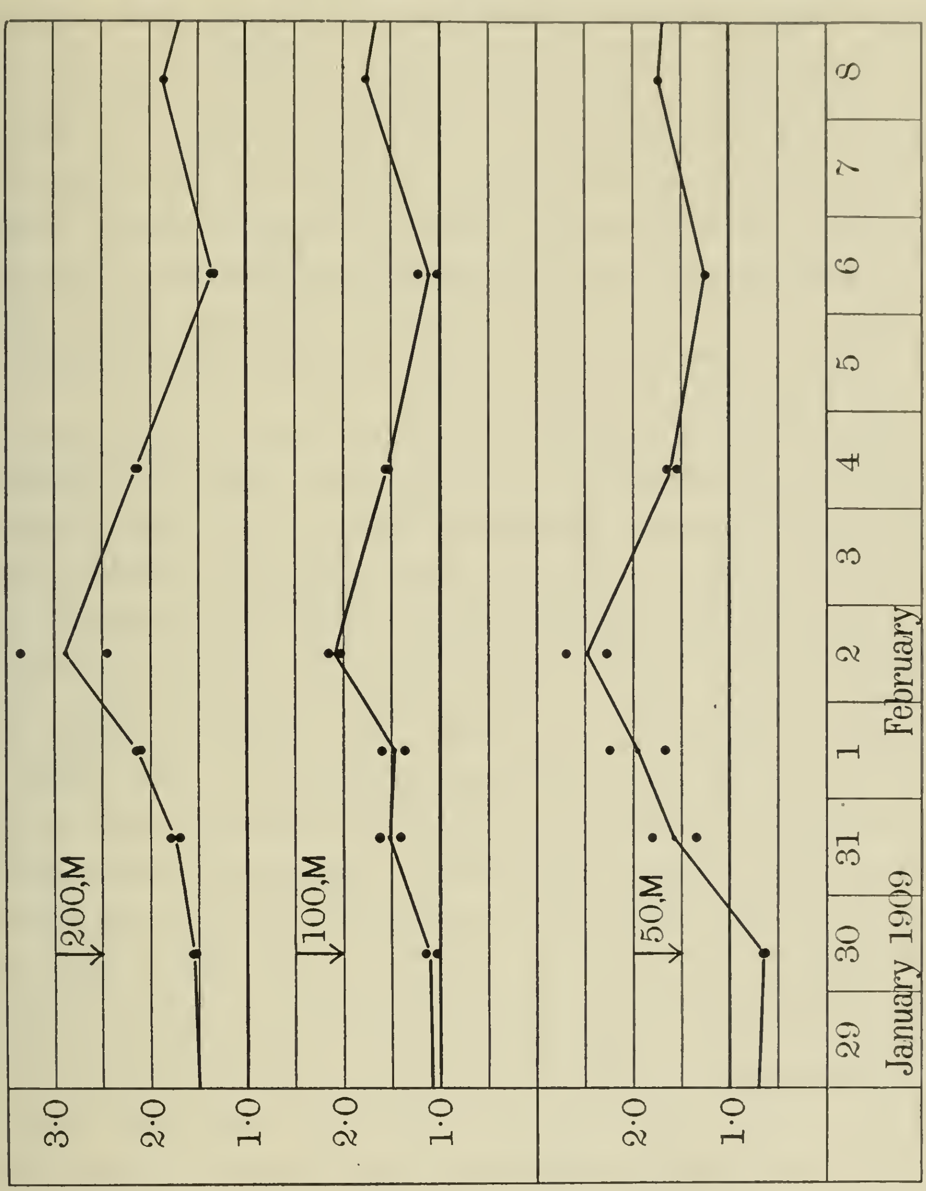


Fig. 50. Rab. 7.

Fig. 51. Rab. 10.

Fig. 52. Rab. 21.

Figs. 50 to 52. Response of rabbits, recovering from a previous infection, to intraperitoneal inoculations of vaccine.

X. CAUSES WHICH TEND TO PREVENT EFFICIENT AUTOINOCULATION IN DISEASE.

The first desideratum, for an efficient reaction, is that it should be prompt. But it has been shown that one tissue differs from another very markedly in the power of prompt response to inoculation, the subcutaneous tissue possessing this power in a high degree, whilst it is absent from the tissues of the peritoneal area. Hence an autoinoculation can only be considered efficient, which affects tissues gifted with this ability to react immediately; and hence also much of the antigen set free within the body will be wasted on tissues of low reactive power. The locality affected by the disease thus becomes of supreme importance, especially in the first stage, before an immunising response has been set up. After the infected animal has acquired a certain degree of immunity, other tissues have been educated to respond readily to the stimulus of inoculation, so that efficient autoinoculation is more readily attained, than it was at the beginning of the disease. But it still remains a matter of chance coincidence if the liberated antigen reaches the tissue best able to react to its presence.

The opsonic charts of rabbits infected by the blood-stream, so far as they go, entirely support this argument. Figs. 23 to 26 refer to rabbits, which suffered from severe generalised infections. In spite of the gravity of the infection, the opsonic charts (Figs. 23, 24, 26) show evidence of the occurrence of remarkably few effective autoinoculations. Rabbit 24. L. indeed, received no effective stimulus at all, in the course of its disease. Rabbit 23. L., during a disease lasting 26 days, only received two autoinoculations; and these were equivalent (judging by the results) to the quantities of vaccine treated of in Part VIII. as "small doses" (Figs. 29, 30, 31). The first, peaked elevation of the opsonic curve, culminating on May 29th, is a typical and active "small dose" response, undisturbed by any succeeding autoinoculation, so that it shows admirably the gradual fall to normal. The second elevation, immediately preceding death, was probably similar in character, but the observations for two days are lacking. Rabbit 25. L. (Fig. 23) also showed a curve indicating small autoinoculations at fairly wide intervals.

About the events of the days intervening between the elevated portions of the curves, one can only deduce that the tissues are receiving a very small amount of bacterial matter. The insignificance of this amount of constant leakage from the foci of infection, is to be argued from its lack of effect as shown on the opsonic charts. But, if it be urged against this view, that we are ignorant of the effect of a constant and diffused supply of bacterial products, bathing the tissues, as it were, in a poisonous medium, then there are two considerations to be met. First, the experiment of inoculating an animal with a large intraperitoneal dose of bacilli, living or dead, shows that the effect of such a bath, applied to only a portion of the whole tissues of the body, is a prompt and marked lowering of the opsonic index (Figs. 15, 16, 19, 20, 21), and consequently such a condition, occurring in disease, should give evidence of its existence upon the opsonic chart. Secondly, if we assume that all the tissues have a constant large supply of bacterial matter to deal with, it is difficult to explain how they can respond so readily, as they certainly do, to the additional stimulus of a small dose of vaccine. It follows that the immunising mechanism of these rabbits only received small and intermittent stimuli from autoinoculation, yet the disease, which could only provide so slight a stimulus, was fatal to two of the three, which presented, post-mortem, convincing indications of a grave septicaemia.

The histological relation of the infecting microbes to the tissues is also of importance, since one condition of stimulation is a close contact between the tissue cell and the bacterial matter. Now most of the bacilli, in this infection, are either enclosed in phagocytes, or shut up in necrotic foci, which later become small abscesses. With regard to the first category, we are ignorant whether a phagocyte can produce specific antibodies, and, if it can, whether these are passed at all into the circulation, or are devoted entirely to the enclosed bacilli, which have excited their formation. With regard to the second category, those bacilli, namely, which are growing within a necrotic area, or in an abscess, consideration will show that they are, for a time, practically shut off from the living tissues of the body. When a bacterial embolus lodges, it can be seen, after a few hours, to be surrounded by a zone of dead cells. As the bacteria multiply and grow into this necrotic mass, the necrosis spreads too, so that the advancing colony is preceded in every direction by this zone of death. Except at the first lodgment of the embolus, therefore, no living tissue has contact with the invaders, only a certain amount of their soluble products diffuses out into living areas. And this, for a time, is the only material available as a stimulus. Later, when the affected area has become limited by an active abscess wall, the contents of which have been liquefied, the conditions are probably much more favourable.

It has been shown, however, by Freeman and others (1907), in the case of localised infections, that their presence may fail to call forth any notable immunising response, until the conditions are altered by massage, active or passive movement, or by induced hyperaemia. These measures insure a sudden disturbance of the infective focus, and may be supposed to bring the infecting bacteria or their products into relation with healthy, or at least active tissues, in suddenly increased quantity. The effect is, undoubtedly, a lively immunising response, which was absent before.

The state of the antigen itself has to be considered, as well as the tissue with which it comes into relation. When a dose of vaccine is given, the antigen is brought into contact, with the tissue elements, all at once, and in considerable concentration. In disease, on the contrary, the antigen is formed gradually by the growth and decay of bacilli spread widely through the animal body. *Pari passu,* with this growth and decay, goes on the absorption of bacteriotropic substances from the circulating fluids, so that each freshly formed bacillus, as well as the products of its growth and decay, is at once partially saturated with antibodies. Such partially saturated antigen constitutes a very much less efficient stimulus to the tissues, than unsaturated antigen, such as that contained in a sterilised vaccine. On this point the experiments of Jorgensen and Madsen (1902) are very clear. These observers found that rabbits and goats, passively immunised by the injection of anti-typhoid sera, would not react to an inoculation of killed typhoid bacilli. But as soon as the passive immunity had passed off, that is, as soon as the agglutination titre of the blood had fallen again to the normal value, the animals would react to inoculations of killed typhoid bacilli, with an energetic production of agglutinin. They argue, from these observations, that, in the case of the passively immunised animals, the inoculated material combined at once with the available antibody, and consequently its stimulating activity became neutralised.

From the arguments set forth above we may perhaps conclude that the causes, which tend to prevent efficient autoinoculation in disease, are the following:—1. The tissues chiefly affected are normally unable to make a prompt specific response to the stimulus which the presence of bacterial matter supplies, such a power being special to the subcutaneous tissues. 2. The *gradual* increase, by growth, of the infecting microbes and their products, which is met, at each step, by a partial saturation with antibodies supplied from the circulating fluids. 3. The temporary shutting up of every large aggregation of bacteria behind a zone of necrotic tissue. 4. (Possibly),—the inclusion of otherwise available stimulus-matter, within the various orders of phagocytes.

CONCLUSIONS.

1. Opsonic immunity is of real importance in determining recovery from an infection with *B. pseudotuberculosis.*

2. The subcutaneous tissue of the rabbit has a special power, not possessed by the peritoneum, of reacting promptly to inoculations of killed cultures of this bacillus, with an increased production of opsonin.

3. The above observation is an instance of the local production of antibodies.

4. Intraperitoneal inoculation of *B. pseudotuberculosis*, living or dead, produces an immunising response after a considerable delay.

5. Intravenous inoculation of the living bacillus, produces an immunising response after a considerable delay.

6. Rabbits previously infected with the bacillus are profoundly altered as regards their reactions towards renewed inoculations of living or killed cultures.

7. Cultures of *B. pseudotuberculosis* killed by heat at 60° C. contain more immediately available antigen, than do equal quantities of living cultures, and hence constitute a more efficient stimulus.

8. The following causes tend to prevent the occurrence of efficient autoinoculation in disease:—The tissues affected may not be reactive; the antigen formed within the body is partially saturated with antibody *pari passu* with its formation; foci of infection are largely shut off from active tissues by necrosis; phagocytosis may prevent the ingested bacilli from functioning as a stimulus.

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ON THE SEASONAL PREVALENCE OF *TRYPANOSOMA LEWISI* IN *MUS RATTUS* AND IN *MUS DECUMANUS* AND ITS RELATION TO THE MECHANISM OF TRANSMISSION OF THE INFECTION.

By G. F. PETRIE, M.D., and C. R. AVARI.

No precise observations so far as we are aware have been published hitherto regarding the seasonal prevalence of any of the infections in man or animals—malaria excepted—which are definitely associated with the presence in the blood of the host of a protozoan. For this reason we have thought it desirable to record an extensive series of observations on the occurrence of *T. lewisi* in *M. rattus* and *M. decumanus* in Bombay made during a period of 14 months in the years 1905—1906.

A recent paper by Nuttall (1908) on the transmission of *T. lewisi* by means of insects has revived interest in the question of the mode of transmission of this parasite from rat to rat. We believe that our observations have a bearing upon this problem and we shall therefore bring under review in due course the epidemiological factors which must be reckoned with in a discussion of the problem.

While reserving full discussion of these factors until later it may be stated at once that evidence will be brought forward which points to a developmental cycle taking place in the insects transmitting the infection. This evidence, derived as it is from purely epidemiological sources, is perhaps of especial interest in view of Kleine's (1909) important experiments on the transmission of *T. brucei* confirmed recently for *T. gambiense* by Bruce (1909)—experiments which make it practically certain that these trypanosomes undergo a cycle of development in the *Glossina palpalis*. Moreover our observations seem to us to shed some fresh light on the transmission of *T. lewisi* and probably also of all insect transmitted trypanosomes in that they give a clue to the dominant factor influencing the cycle of development.

It is somewhat curious perhaps that notwithstanding the large amount of work devoted to the biology of *T. lewisi*—work which has aided materially in guiding researches upon the pathogenic trypanosomes—very scanty attention appears to have been paid to the question of possible seasonal variations in the prevalence of the infection amongst rats. Before bringing forward our own observations we may briefly cite references to the subject by other writers.

It is interesting to note that Vandyke Carter (1887) who in 1885 first observed the occurrence of *T. lewisi* in Bombay rats (chiefly *M. decumanus* and *M. rattus* (?)) clearly recognised the possibility of a seasonal prevalence of the infection. Thus Carter states in his memoir that from his observations—referring presumably to their paucity—"valid inference regarding a possible seasonal prevalence could not be made." Again he writes: "In the same connection I would here allude to the evident narrow place-limitation or endemicity of both rat infection and the 'surra' disease with probably also a distinct seasonal variation of prevalence."

Lingard (1895) examined a large number of rats (*M. decumanus*) in Bombay during several years and is quoted by Laveran and Mesnil in their book on trypanosomes as having found that during the rainy season, June to October, 42 °/₀ of the rats were infected with trypanosomes, while during the dry season, November to May, only 28 °/₀ were infected. These observations of Lingard's will be referred to at greater length below.

Musgrave and Clegg (1903) remark that in Manila rats (thousands examined) *T. lewisi* has been found in from 20—65 °/₀ of the individuals examined, varying according to the season and to the locality from which they were received. Unfortunately these authors content themselves with this bare statement—details are absolutely lacking.

Swingle (1907) recites a few facts from his own experience in Nebraska which seem to indicate a seasonal prevalence of the infection. "Of the 17 one-fourth grown rats examined in the autumn and winter not one was infected with either lice, fleas or trypanosomes, while of the seven caught the following spring at the same place all had fleas and four were harbouring the parasite."

Lastly, Yakimoff (1907) is cited by Mesnil as stating that in an investigation of the distribution of the infection amongst the rats in St Petersburg the proportion of rats infected was greater in the warm than in the cold season.

The present series of observations was begun on 1st September, 1905, and was continued without intermission until 19th October, 1906, a period of nearly 14 months[1]. The rats were brought from all parts of Bombay city and island for the purposes of the Plague Commission then working at the laboratory at Parel. The observations were made generally on five or six days of each week. Only "young" rats were examined for the presence of trypanosomes, i.e. in accordance with the arbitrary standard adopted by the Commission, those weighing 70 and 100 grammes or less in the case of *M. rattus* and *M. decumanus* respectively; no rats below these weights were found pregnant. Young rats were selected with the idea of eliminating the factor of immunity acquired in adult rats either by their having harboured the infection at some previous time or by vaccination with doses of infected blood insufficient to bring about an infection. When brought to the laboratory the young "live" rats were killed and at once dissected. Blood was pipetted from the heart and spread in a somewhat thick layer upon clean slides. The blood films after fixation with absolute alcohol were stained with carbol-thionin and examined under an oil immersion lens. It was found that carbol-thionin had the advantage as a diagnostic stain for trypanosomes of staining the red blood corpuscles very feebly so that a film of moderate thickness could be searched for the parasites without difficulty.

In all, 1832 *M. decumanus* and 2651 *M. rattus*, a total of 4483 rats, were examined during the period of investigation. The results are shown in the tables (I, II and III) and in Chart I. It will be noted (Tables I, II and III) that, as might be expected, there is no marked difference in the incidence of the infection on males and females of both species. Table III also shows that the percentage of *M. rattus* infected during the whole period does not differ markedly from the percentage of infected *M. decumanus*.

It is interesting to compare these results with those obtained by previous observers in Bombay. Vandyke Carter in 1885 examined 210 rats for trypanosomes and found 25 infected, i.e. nearly 12 %. From November 1890, to May 1895, Lingard in the course of his work on

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[1] We may note that the primary object we had in view in undertaking the investigation was in order to determine if possible, a seasonal prevalence of trypanosomes in rats which might give a clue to the season of rat-flea prevalence, an important question in plague epidemiology. In this we were influenced by the experiments of Rabinowitsch and Kempner (1899) which seemed to warrant the belief that rat fleas could act as transmitters of the infection in nature. As it happened the Plague Commission were able to obtain direct evidence during the year subsequent to our investigations as to a season of rat flea prevalence in Bombay.

TABLE I.

Giving results of examination of M. rattus *for trypanosomes.*

Monthly periods	Males			Females			Total *		
	Examined	Infected	Percentage	Examined	Infected	Percentage	Examined	Infected	Percentage
September 1905	97	38	39·2	117	41	35·0	302	106	35·0
October	137	71	51·8	114	61	53·5	251	132	52·6
November	101	48	47·5	80	44	55·0	191	94	49·2
December	121	58	47·9	118	54	45·8	239	112	46·9
January 1906	116	55	47·4	131	42	32·1	247	98	39·7
February	82	29	35·4	93	21	22·6	175	50	28·6
March	69	10	14·5	78	14	17·9	147	24	16·3
April	68	16	23·5	69	13	18·8	137	28	20·4
May	42	11	26·2	50	16	32·0	92	27	29·3
June	118	59	50·0	89	55	61·8	207	114	55·0
July	93	58	62·4	81	44	54·3	174	102	58·6
August	84	51	60·7	112	74	66·0	196	125	63·8
September	73	45	61·6	84	48	57·1	157	93	59·2
October	72	43	59·7	64	40	62·5	136	83	61·0

* These columns include a number of rats in which the sex was not noted.

TABLE II.

Giving results of examination of M. decumanus *for trypanosomes.*

Monthly periods	Males			Females			Total *		
	Examined	Infected	Percentage	Examined	Infected	Percentage	Examined	Infected	Percentage
September 1905	59	32	54·2	66	29	43·9	196	70	35·7
October	128	43	33·6	101	33	32·6	229	76	33·2
November	81	37	45·7	72	44	61·1	183	87	47·5
December	94	49	52·1	94	44	44·4	208	93	44·7
January 1906	85	39	45·9	110	46	41·8	219	85	38·8
February	49	7	14·3	63	17	27·0	112	24	21·4
March	52	6	11·5	43	14	32·5	95	20	21·0
April	35	2	5·7	39	3	7·7	77	5	6·5
May	8	1	12·5	10	2	20·0	18	3	16·6
June	55	29	52·7	47	22	46·8	102	51	50·0
July	61	25	41·0	52	32	61·5	113	57	50·4
August	52	26	50·0	60	35	58·3	112	61	54·5
September	40	22	55·0	34	16	47·0	74	38	51·4
October	47	28	59·5	47	26	55·3	94	54	57·4

* These columns include a number of rats in which the sex was not noted.

TABLE III.

Summarising results of examination of M. decumanus *and* M. rattus *for trypanosomes.*

M. decumanus			*M. rattus*		
Males examined	= 846	40·9 °/₀	Males examined	= 1273	46·5 °/₀
Males infected	= 346		Males infected	= 592	
Females examined	= 838	43·3 °/₀	Females examined	= 1280	44·3 °/₀
Females infected	= 363		Females infected	= 567	
Total* examined	= 1832	39·5 °/₀	Total* examined	= 2651	44·8 °/₀
Total infected	= 724		Total infected	= 1188	

* Includes a number of rats in which the sex was not noted.

Surra examined 3105 *M. decumanus* and found 35 °/₀ infected. Our observations show that in 1905—1906, 40—45 °/₀ of the Bombay rats were infected. These results taken as a whole appear to confirm the conclusion arrived at by Laveran and Mesnil on experimental grounds in their work on *T. lewisi* that "immunization through the placenta or by lactation is exceptional if it occurs at all." At least the figures give no ground for belief that during a period of 20 years the rat population in Bombay has become less susceptible as the result of a cumulative hereditary transmission of immunity against the infection.

Coming now to the consideration of the question of the seasonal prevalence of the infection, it is clear from the details given in the tables and represented graphically on the chart that a marked seasonal variation in prevalence exists, and that the curves of prevalence for *M. rattus* and *M. decumanus* present a noteworthy resemblance. Thus in the months June to December the percentage figures of infected animals of both species rise above the mean for the year, while in the remaining months the percentage figures fall below the mean. The lowest percentages of infected animals are found in March and April, corresponding to 6·5 °/₀ infected *M. decumanus* and 16·3 °/₀ infected *M. rattus*, while the highest percentages appear from the chart to correspond to the month of August, in which 54·5 °/₀ *M. decumanus* and 63·8 °/₀ *M. rattus* proved to be infected.

During the preparation of this paper for publication we have obtained a striking confirmation of these results while consulting Lingard's original report (1895) of his observations on *T. lewisi* in Bombay. Lingard in this report (*Summary of Further Report on Surra*, Bombay 1895) gives the data which led him to the conclusion that a larger percentage of rats are infected during the months June to October than during the remaining months of the year. His percentage figures of infected animals (*M. decumanus*) for each month from May 1894, to April 1895 are reproduced in Table IV; the total number of animals examined during this year was 2102. We have represented these figures in Chart II in the same manner as has been done in Chart I, and it will be seen that the prevalence curve based on Lingard's figures approximates very nearly to our own curves[1].

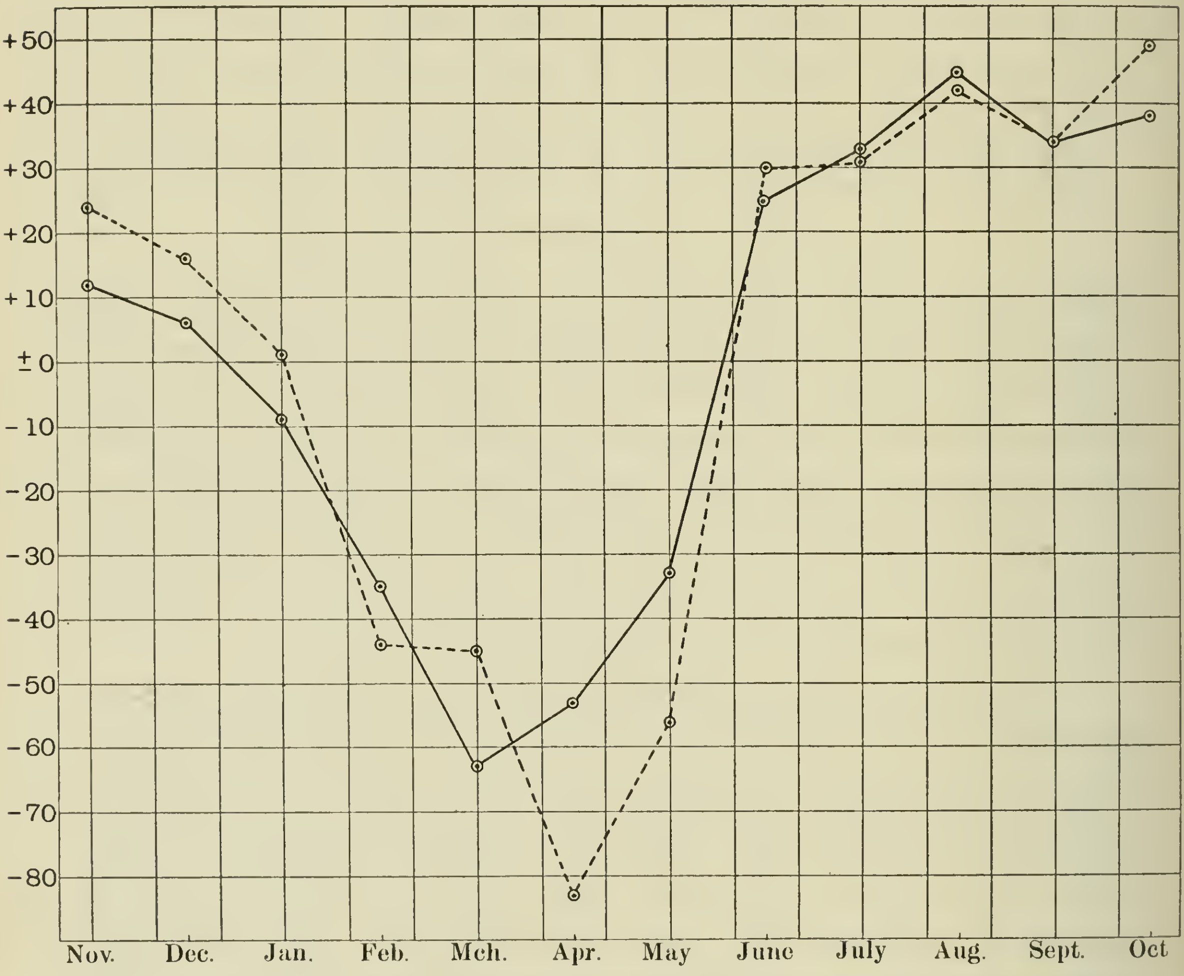

Chart I. Prevalence of trypanosomes in *Mus rattus* and *Mus decumanus*, Bombay, 1905, 1906.

——— = *Mus rattus.*
- - - - - = *Mus decumanus.*

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[1] For convenience of comparison the months in Chart II are arranged in an order which is not entirely consecutive, but this does not appear to us to invalidate a general comparison between the two charts.

Lingard apparently examined all rats available without reference to age.

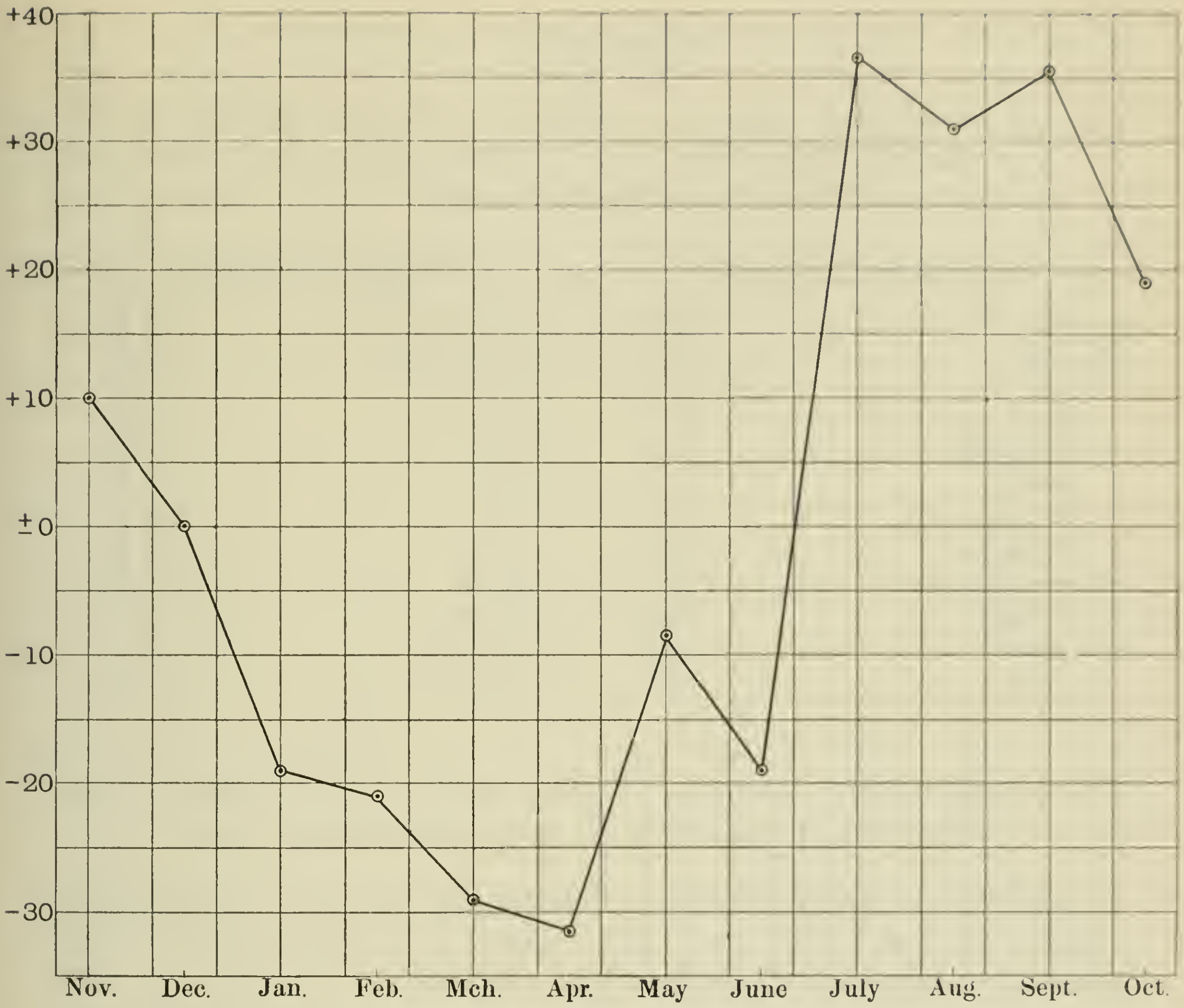

Chart II. Showing prevalence of trypanosomes in *Mus decumanus*, Bombay, 1894, 1895. (Constructed from Lingard's figures.)

With regard to the interpretation of these curves we cannot but believe that this is bound up with the mechanism of transmission of the infection. The present state of knowledge on this subject does not justify us in bringing forward any positive conclusions in the matter. Nevertheless some considerations may be advanced here which in our view assist in explaining the facts narrated above.

If it be admitted that the explanation of the variations in prevalence is probably to be found in the mode of transmission of the parasite, two hypotheses suggest themselves as affording a reasonable interpretation of the facts. The first hypothesis is that the trypanosome prevalence is associated with a seasonal prevalence of the insects transmitting the infection, and the second possibility is that apart from insect prevalence the conditions for successful transmission of the infection by insects vary at different seasons of the year.

TABLE IV.

Showing prevalence of trypanosome infections in M. decumanus (*abstracted from Lingard's Reports*) *and of Adie's* Leucocytozoon *in* M. rattus (*abstracted from Plague Commission's Reports*).

Percentage of *M. decumanus* infected with trypanosomes		Percentage of *M. rattus* infected with leucocytozoon	
May 1894	31·2	December 1905	16·8
June	27·5	January 1906	14·6
July	46·7	February	7·1
August	43·0	March	4·1
September	46·3	April	0·7
October	40·4	May	5·4
November	37·5	June	22·4
December	34·1	July	21·7
January 1895	27·5	August	21·6
February	26·6	September	19·2
March	24·1	October	16·7
April	23·4	November	15·8

The question of the correlation of the prevalence of the infection with the seasonal prevalence of the ectoparasites of rats.

The insects which infest the Bombay rats are fleas (*Loemopsylla cheopis*, Rothsch.) and lice. With regard to the prevalence of rat fleas in Bombay the extensive data collected and published by the Plague Research Commission (1908) are happily available for comparison. It seems scarcely necessary to point out that rat fleas are very abundant on the rats in Bombay. The Commission found that the average number of fleas per rat taken from *M. rattus* during a year's investigation was 3·7, while the average number per rat in *M. decumanus* was 8·5. They further determined that there is a definite seasonal prevalence of rat fleas in Bombay. The curves of rat flea prevalence are reproduced from the Commission's reports in Chart III, from which it will be seen that the highest point of the curve of fleas taken from *M. rattus* falls in March and April and corresponds to an average per rat for these months of 5·2 fleas, while the lowest point is found in November, corresponding with an average of 2·5 fleas per rat. The highest point in the *M. decumanus* curve corresponds to an average of 14 fleas per rat in April and the lowest point to an average of 4·2 per rat in September. When a comparison is instituted between the rat flea curves of prevalence and the curves representing the prevalence of trypanosomes it is at once obvious that the curves in the two charts show a completely inverse relation—the off season in the one case corresponding to the season of prevalence in the other.

Before attempting to compare these charts from the point of view of a possible causal relationship, it is necessary to take into account the mean interval elapsing between the date of infection of the rats and the date on which examination revealed the parasites in their blood. It is impossible to determine this interval with any degree of accuracy, but it may be pointed out that all the rats examined were young, probably on an average 4—6 weeks old. It may be noted also in this connection that Laveran and Mesnil (1901) have found experimentally that in young rats (30—100 grammes) the incubation period, i.e. the interval between inoculation of infected blood and the appearance of the parasites in the circulation, is very short—usually only 24 hours. For these reasons we think that a general comparison may justly be made between

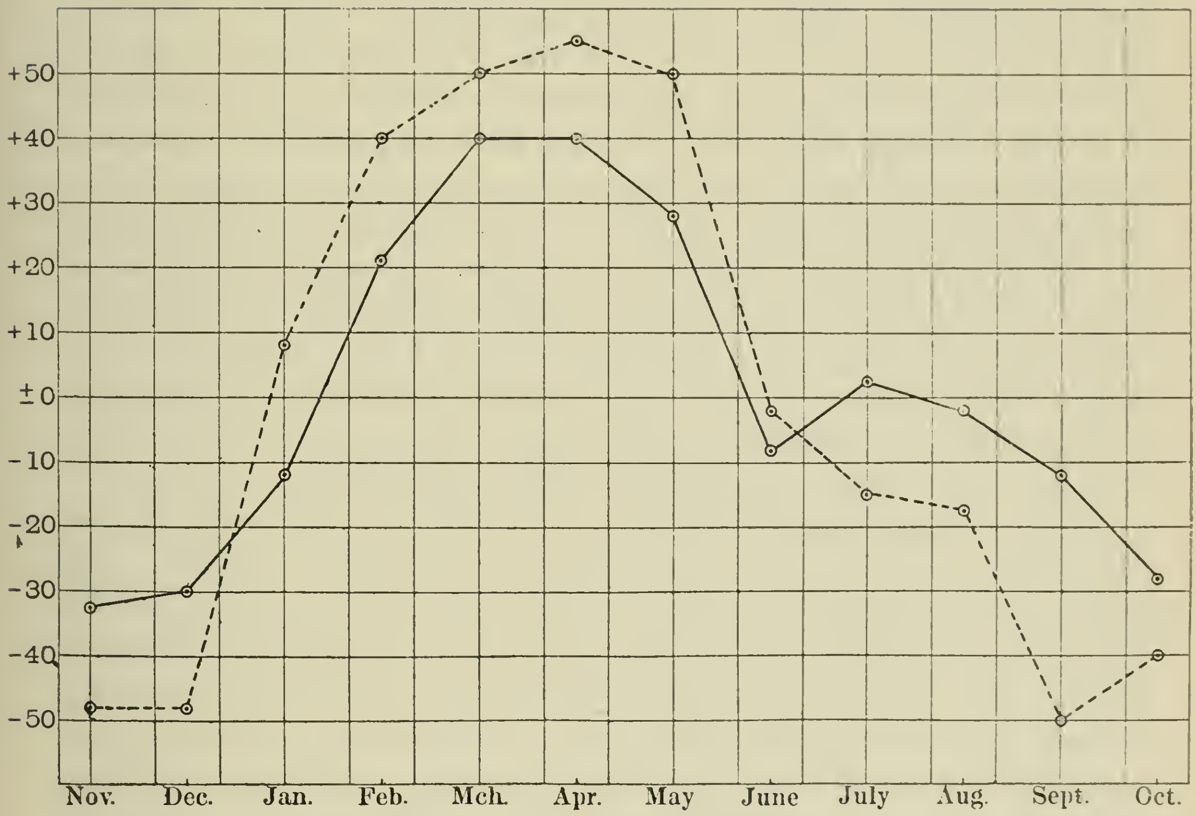

Chart III. Showing Rat flea prevalence in Bombay, 1906, 1907. (From the Plague Commission's Reports, *Journ. Hygiene*, Vol. VIII.)
——— = Fleas on *Mus rattus.*
- - - - - = Fleas on *Mus decumanus.*

the two charts. Must it be supposed then, as at first sight it appears, that there is no relation between the prevalence of rat fleas in Bombay and the occurrence of trypanosomes in the rats? It cannot be doubted that if rat fleas can act as transmitters of *T. lewisi* the propagation of the infection amongst the rats must be aided by a seasonal increase in the number of rat fleas. The Plague Commission, for example, have shown that the rat flea prevalence in Bombay is associated in a marked manner with the prevalence of plague amongst the rats. It is difficult, in view of Nuttall's (1908) recent transmission experiments with rat fleas (*Ceratophyllus fasciatus* and *Ctenophthalmus agyrtes*), to suppose that *Loemopsylla cheopis* is incapable of conveying the infection. Moreover certain observations of our own in Bombay strongly suggest that the latter insect is able to originate an outbreak of the infection. A batch of tame white rats sent out from England were confined together in a cage which was deposited on the floor of a room in the laboratory; this room was over-run with *M. rattus* and *M. decumanus,* and needless to say was infested with rat fleas. After a brief interval a number of the English rats died and trypanosomes were found in their blood; a few rat fleas were captured on some of these rats. Now the construction of the cage was such that rat fleas on the floor of the room might readily gain entrance into the cage, whereas transference of lice from the wild to the tame rats did not seem at all likely.

There is one point which deserves attention in a discussion of the relation between the prevalence of trypanosomes and of rat fleas. As the Plague Commission remark in their report, fleas are most abundantly found in the haunts of their hosts owing to the fact that the houses or nests of the host are *par excellence* the breeding places of these insects. It may be therefore that owing to the "concentration" of fleas in the nests and burrows of the rats the opportunities for infection of young rats tend as it were to be levelled up. This explanation is not, however, wholly adequate, and there remains therefore the supposition that during the season of maximum prevalence of trypanosome infections other factors in the successful transmission of the infection are operative in spite of a diminution in numbers of the transmitting insects and that similarly during the off season of trypanosome infections these factors are partially in abeyance, so that the influence of an increase in the numbers of the transmitting insects is neutralised.

In connection with the question of the infection of young rats in their nests the figures given in the accompanying table (V) were worked out with the idea that they might establish a relation between the age as judged by the weight of the rats and the degree of infection in the blood. It appears, however, that the difference in weight of the various lots of rats taken is not sufficiently marked to justify any conclusion on this point.

TABLE V.

Comparing the degree of infection with the average weight of M. rattus *and* M. decumanus *infected with trypanosomes.*

Mus rattus.			*Mus decumanus.*		
Degree of infection	Total rats examined	Average weight of rats examined	Degree of infection	Total rats examined	Average weight of rats examined
+	107	47·6 grammes	+	80	62·9 grammes
+ +	86	59·1 ,,	+ +	68	63·8
+ + +	134	58·0 ,,	+ + +	81	58·4

Note :—+ = few, + + = fairly numerous, + + + = numerous or very numerous.

No systematic observations have yet been carried out on the prevalence of rat lice in Bombay. In view of Nuttall's (1908) successful transmission experiments with rat lice such observations would be of interest. From general observations, however, it is fairly certain that the season of prevalence of various insect parasites (human-, rat-, cat-fleas and bugs) is the same for all, so that it is probable that the rat flea prevalence curves provide a fair index of the prevalence of rat lice.

Reference may be made here to the relative importance from an epidemiological standpoint of the rat flea and the rat louse in the transmission of *T. lewisi*, assuming that both are capable of transferring the infection from rat to rat in nature. There would seem to be little doubt that the habits of these insects in relation to their host are important in this connection. Thus lice are well known to live constantly upon their host, and this habit must necessarily limit the opportunities for conveyance of infection from rat to rat, although it is readily conceivable that young rats while in the nest, and especially while suckling, might pick up infected lice. Rat fleas on the other hand, from the fact that they spend much of their existence apart from their host, are more likely to convey the infection from rat to rat over considerable distances.

Interpretation of the trypanosome prevalence curves on the view that the conditions for successful transmission vary at different seasons of the year.

It is conceivable that the climatic conditions at a particular season of the year are especially favourable in respect of the persistence or development of the trypanosomes in the transmitting insect, thereby increasing the chances of successful transmission to the vertebrate host. It is extremely likely for example that if the trypanosomes undergo a developmental cycle in the flea or louse there is an optimum temperature at which such development proceeds. It will be remembered that Grassi (1906) and others have shown experimentally that there is an optimum temperature (77°—86° F.) for sporogony of the malarial parasite in the mosquito, and that Ruge (1906) has given similar proof of the influence of temperature in the case of the *Proteosoma* of birds. Up to the present time, however, developmental stages of *T. lewisi* have not been described in the rat flea, and the observations of Prowazek (1905) on the development of *T. lewisi* in the rat louse have not been accepted by other workers as established.

In Chart IV the temperature curve[1] for Bombay is presented for the year under review together with the trypanosome curve for *M. rattus*[2]; the mean monthly temperature is 79° F.

It will be noticed that the trypanosome curve closely follows the temperature curve. This relationship is indeed so marked as in our view strongly to suggest that temperature is an important factor in determining the prevalence of trypanosome infections in rats. Moreover, since temperature can conceivably exercise an influence only by its effect upon the development of the trypanosomes in the transmitting insects, the relationship between temperature and trypanosome prevalence must be regarded as explicable on this theory. As to the exact significance of the interval separating the two curves it would be premature at present to speculate.

There is yet another influence, however, which climatic conditions may have upon the life history of *T. lewisi* in the agents of transmission. Judging from present knowledge of the conveyance of trypanosomes from insects to the vertebrate host it would seem very probable that in the case we are considering direct or mechanical transference of the trypanosomes takes place. If this is so—and future experiments will doubtless settle the point—it must be supposed that an important

[1] The curves in Charts IV, V and VIII have been constructed by taking the mean temperature and the mean humidity for the year and expressing the respective deviations in each month as percentages in terms of the mean. In Chart VII the "mean line" corresponds to a hypothetical optimum temperature of 79° F., the monthly deviations being grouped in relation to the "mean line" as percentages in terms of the optimum temperature.

[2] The *rattus* curve has been chosen in preference to the *decumanus* curve as being based on larger figures and therefore probably more accurate.

factor in the successful conveyance of infection is the length of time the trypanosomes persist in an infective condition in the region of the contaminated mouth-parts of the transmitting insect. Some experiments bearing upon this question may be quoted here which illustrate the effect of drying, on the infectivity of blood containing trypanosomes. Bruce (1897) in his classical Nagana work, tested the point in an ingenious way by taking threads which had been dipped in blood containing the haematozoa; these were threaded at varying periods of time by a needle under the skin of healthy dogs. Bruce reported that these experiments "would go to show that the blood of animals affected by fly disease retains its capability of transmitting the disease in a dried condition for 24 hours, but that this is exceptional and that at the end of 48 hours the blood is inert." Bruce further states that he has seen living trypanosomes and red blood corpuscles in the proboscis up to 46 hours after feeding. Kanthack, Durham and Blandford (1899) found

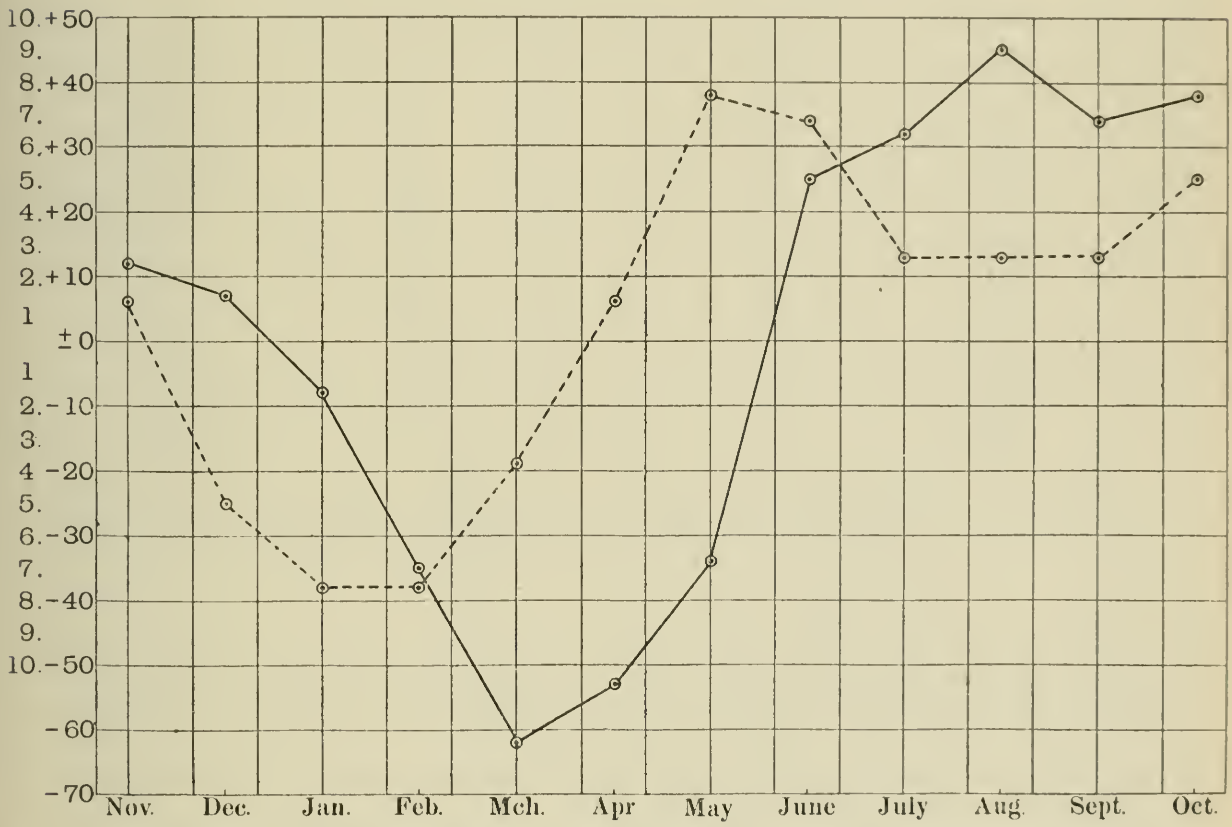

Chart IV. Showing relation of prevalence of trypanosomes to temperature.

——— = Trypanosomes in *M. rattus.*

- - - - - = Temperature.

Mean temperature = 79° F.

that complete drying of blood containing *T. brucei* rendered the blood non-infective. Examination of Chart V from this point of view makes it clear that speaking generally the period of least prevalence of trypanosome infections in rats corresponds to the season of the year during which the humidity is below the mean for the year, viz. 75 (saturation = 100) while the period of greatest prevalence of trypanosome infections corresponds to the season during which the humidity is above the mean[1].

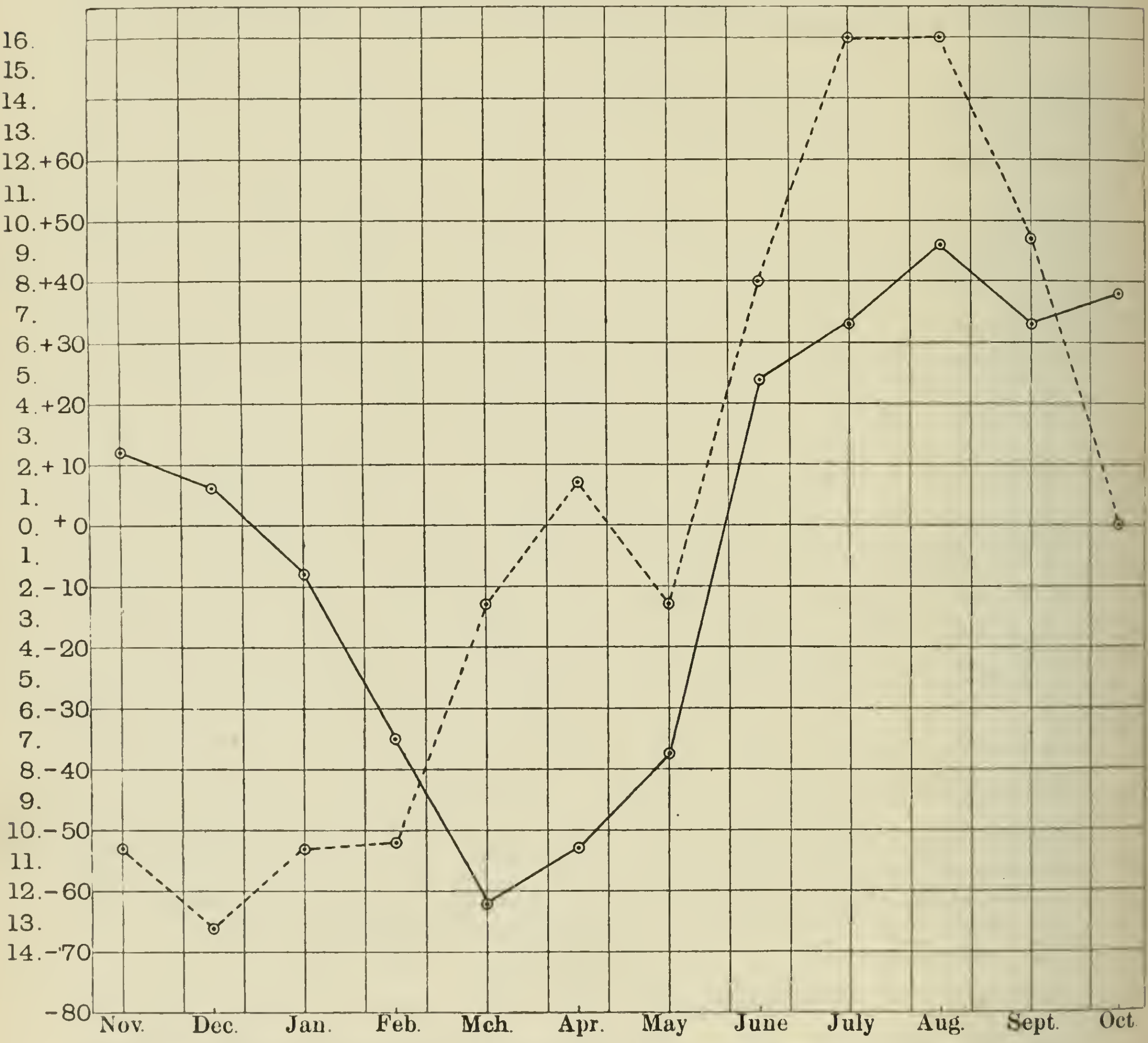

Chart V. Showing the relation of trypanosome prevalence in *Mus rattus* to atmospheric humidity.

——— = *T. lewisi* in *Mus rattus*.

- - - - - = Humidity (the mean = 75°).

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[1] The rainy season in Bombay extends from June to October; the cold dry season comprising the months November to March, and the hot dry season the months April to the middle of June.

It would seem, however, that this relationship is not so striking as in the case of the temperature.

TABLE VI.

Giving mean monthly figures for temperature and humidity (saturation = 100) *for Bombay and Lahore, Punjab**, 1905—1906.

	Mean temperature.		Mean humidity.	
	Bombay	Lahore	Bombay	Lahore
November 1905	80	—	67	—
December	75	57	65	89
January 1906	73	54	67	84
February	73	57	67	88
March	76	65	73	81
April	80	76	76	51
May	85	88	73	39
June	84	94	81	57
July	81	93	87	69
August	81	89	87	74
September	81	84	82	77
October	83	77	75	67
November	—	69	—	78

* The 2 Punjab villages, the rats of which were examined for the *Leucocytozoon*, are situated only a few miles from Lahore.

It has been shown above that in the case of *T. lewisi* in rats definite seasonal variations in prevalence exist. Before concluding this paper another example may be given of a "chronic" protozoal infection in rats showing similar seasonal variations. The parasite to which we refer is the *Leucocytozoon*, which was commonly observed by the Plague Research Commission (1907) in the rats (*M. rattus*) of two Punjab villages. A daily record was kept of the number of rats, in the spleen of which this parasite was found. Altogether during a year's investigation 9499 rats were searched for the parasite by the Commission and 1309 rats were found infected, a percentage for the entire year of 13·8.

We have represented graphically in Chart VI (see also Table IV) the monthly percentage figures of infected animals published in the Commission's reports. It is apparent that the prevalence curve of the *Leucocytozoon* bears a general resemblance to the curves representing the prevalence of *T. lewisi* in Bombay. It will be noted that in this case also no definite relation appears to exist between the *Leucocytozoon* prevalence and the flea prevalence[1].

Chart VII, showing the relation of temperature to the *Leucocytozoon* prevalence, appears to us to furnish similar indications to those discussed above.

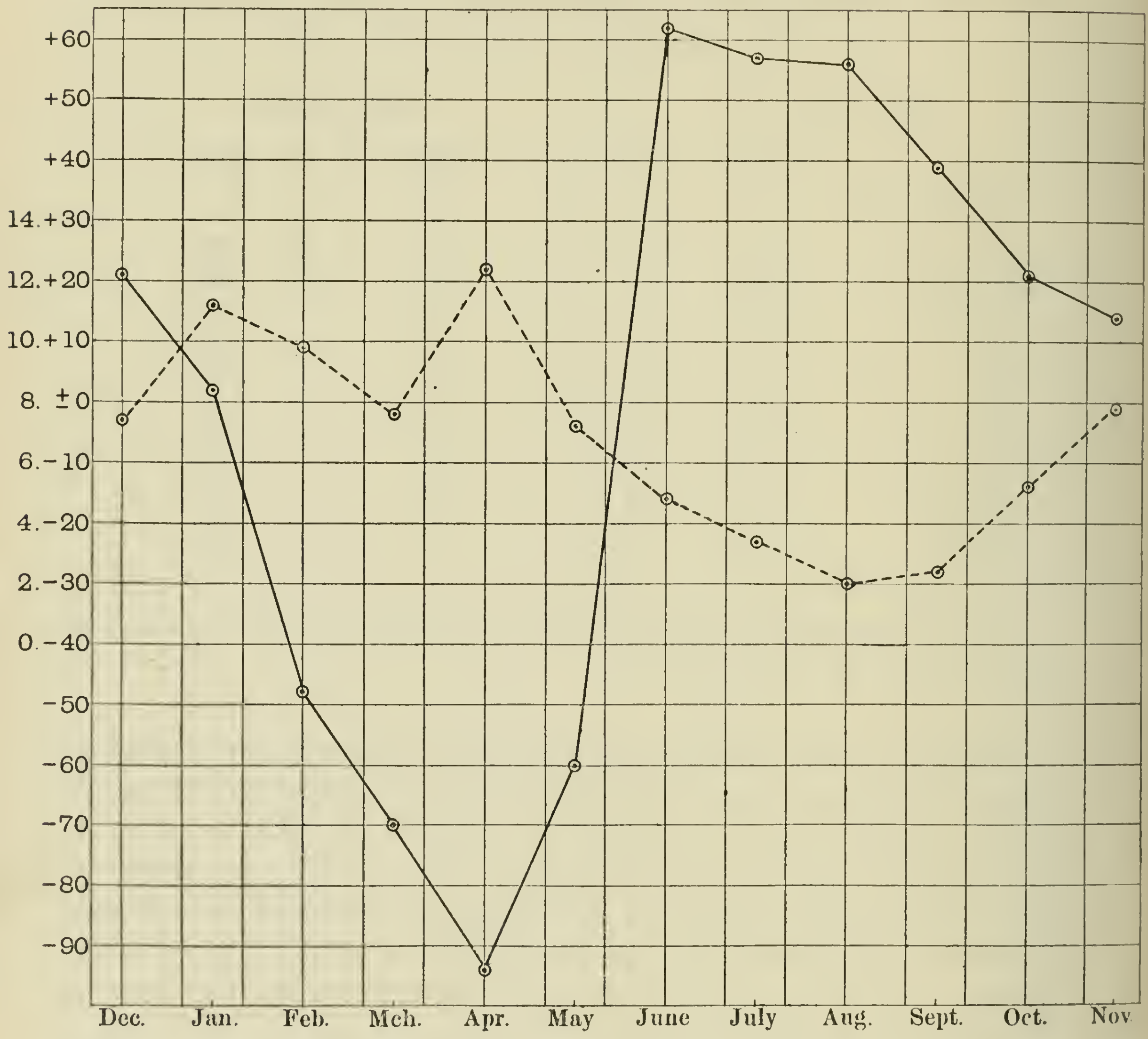

Chart VI. Prevalence of *Leucocytozoon* in *Mus rattus* in Punjab (constructed from Plague Commission's figures) and its relation to Rat flea prevalence, 1905, 1906.
——— = Leucocytozoon prevalence.
- - - - - = Rat flea prevalence (from Plague Commission's Reports).

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[1] The fleas were taken from the rats examined for the *Leucocytozoon*.

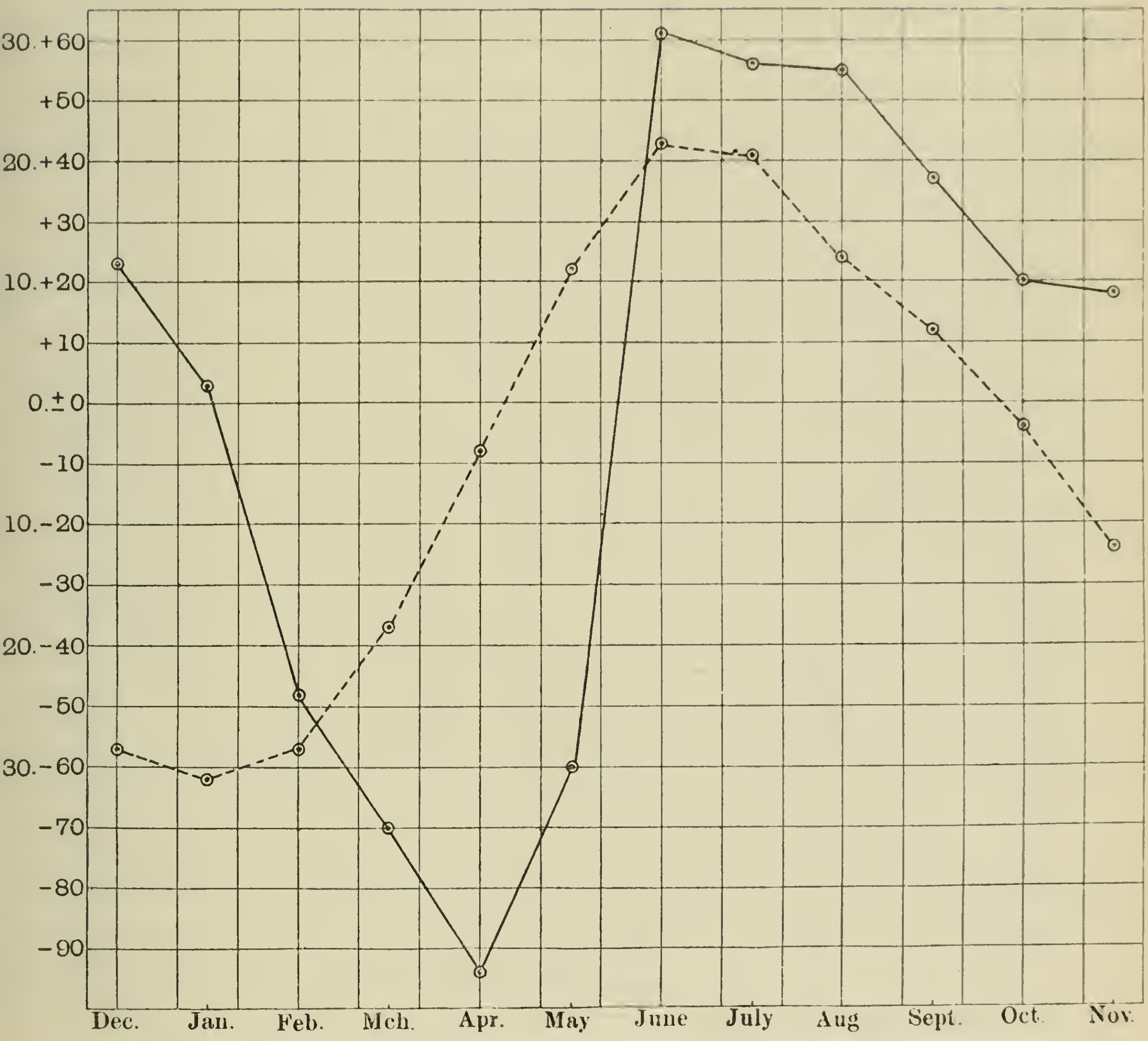

Chart VII. Showing relation of the prevalence of *Leucocytozoon* to temperature.

——— = *Leucocytozoon* prevalence.

- - - - - = Temperature.

Mean temperature = 79° F.

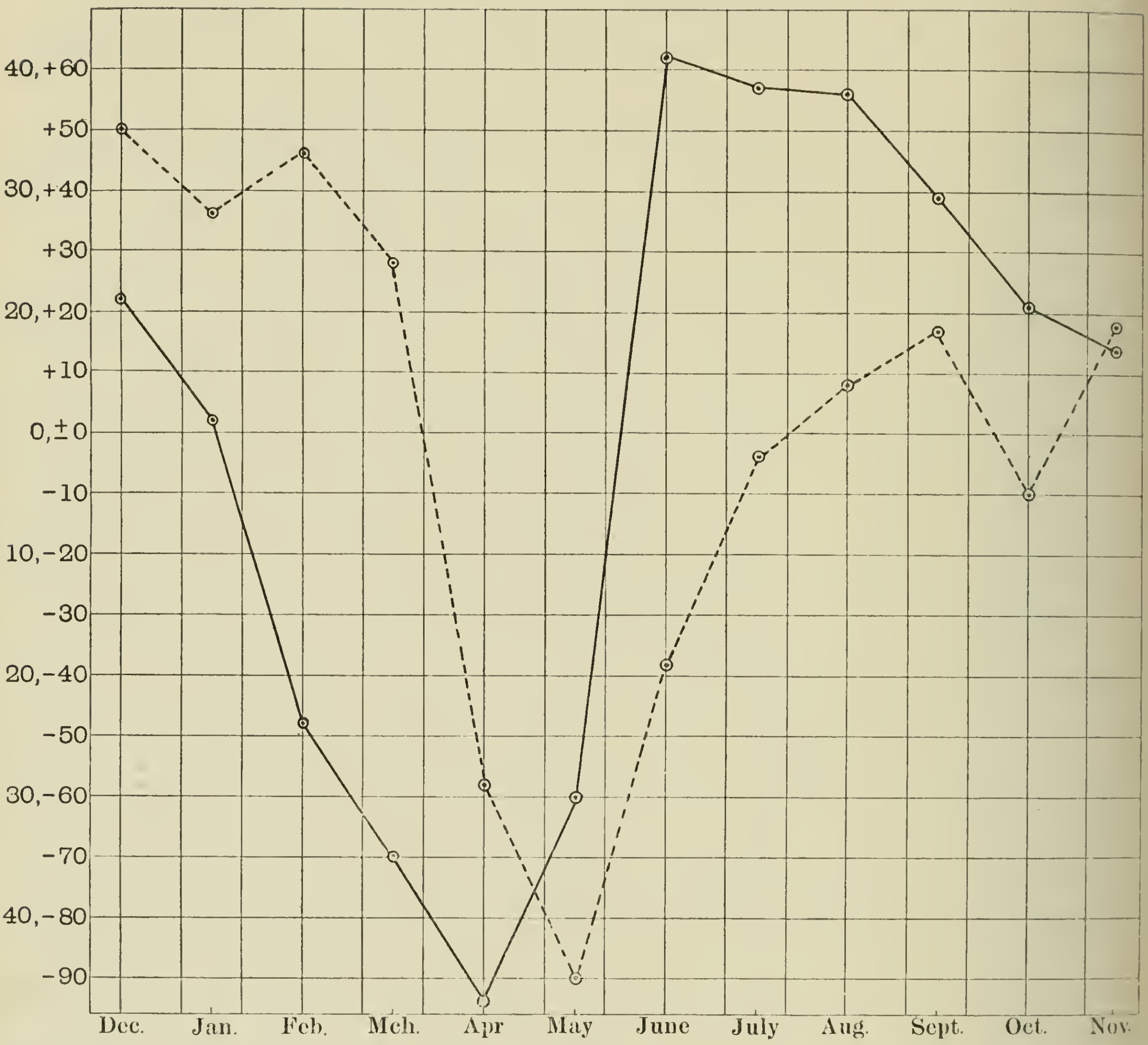

Chart VIII. Showing relation of *Leucocytozoon* to atmospheric humidity.
——=*Leucocytozoon* prevalence.
-----=Humidity.

Summary of Conclusions.

The above-mentioned considerations bearing upon the influence of insect prevalence and the influence of meteorological conditions on the prevalence of trypanosome infections in rats may be summarized as follows:

(1) There are definite seasonal variations in the prevalence of trypanosome infections in *M. rattus* and in *M. decumanus.*

(2) No definite correlation is forthcoming between the seasonal prevalence of *T. lewisi* and the seasonal prevalence of rat fleas. The seasonal prevalence of the transmitting insects is apparently a factor of subsidiary importance in the causation of the trypanosome prevalence.

(3) The evidence adduced above indicates that the dominant factor determining the seasonal prevalence of trypanosome infections is the atmospheric temperature, the optimum temperature approximating to 79° F. It is probable that temperature operates by influencing a developmental cycle of the trypanosomes in the transmitting insects.

(4) It is possible that in addition to temperature the atmospheric humidity may play a part by influencing the direct or mechanical transference of the infection.

It may be added in conclusion that the considerations discussed above seem to us to indicate strongly that transmission experiments with *T. lewisi* (and probably other trypanosomes) will give the best chance of success if carried out during the season of greatest prevalence of the natural infection, and further that in experimental investigations of the development of trypanosomes—pathogenic or non-pathogenic—in insects transmitting the infection to man or animals, the temperature at which the experiments are carried out should be looked upon as of the greatest importance.

Moreover, since it has been shown above that the seasonal prevalence of another parasite of the rat (a *Leucocytozoon*) bears a similar relation to temperature it is probable that the statement just made is a generalisation which will be found applicable to many protozoal infections transmitted by the agency of insects.

We have pleasure in recording our indebtedness to Lt.-Col. W. B. Bannerman, M.D., I.M.S., Director of the Bombay Plague Research Laboratory at the time these observations were made, and to Dr V. L. Manker, Mr P. S. Ramachandrier, Mr Mallet and Mr Sabnis for assistance rendered to us in various ways.

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[*From the* PROCEEDINGS OF THE ZOOLOGICAL SOCIETY OF LONDON, 1909.]
[Published June 1909.]

Report on Deaths which occurred in the Zoological Gardens during 1908. By H. G. PLIMMER, F.L.S., F.Z.S., Pathologist to the Society.

On January 1, 1908, the number of animals in the Zoological Gardens was 3190 and during the year there were 2418 new arrivals, making a total for the year of 5608. 1737 animals have died during the year, that is roughly 31 per cent., but if from this number we subtract 769 animals which did not live for six months after their arrival in the Gardens—that is, those which had not got used to their new environment—the percentage of deaths is reduced to 17 per cent., which is probably a much fairer number to take.

Of those which have died, 1089 have been examined, and in 41 of these no cause of death could be found. Of the remainder 129 died from injuries of various sorts, or were lost, or not sent for examination, and 30 died from exhaustion due probably to depressed vitality from cold or darkness, or from inability to get or to take food.

The Tables which follow show the facts ascertained in bare outline, and following them are some short notes on the most important points connected with these facts.

Table I. sets forth the causes of death in each of the three great classes of animals. Under Reptiles are included batrachians and fishes.

TABLE I.—Analysis of 993 deaths.

DISEASE.	MAMMALS.	BIRDS.	REPTILES.	
1. *Microbic or Parasitic Diseases.*				*See Notes.*
Tuberculosis	59	88	17	1.
Mycosis	.....	55	......	2.
Malaria	......	14	......	3.
Filaria	3	18	1	4.
Trypanosomiasis	1	2	......	5.
Hæmogregarines	......	......	24	6.
Pneumonia	47	29	19	7.
Septicæmia	12	1	......	
Pseudo-tuberculosis	3	......	......	
Hydatids	1	......	......	
Worms	3	......	15	
Itch	3	......	......	
Protozoal infection	......	......	1	8.
2. *Diseases of Lungs.*				
Broncho-pneumonia	44	......	......	
Congestion of lungs	29	100	14	9.
3. *Diseases of Heart.*				
Pericarditis	7	3	1	
Fatty degeneration	.....	3	......	
4. *Diseases of Liver.*				
Hepatitis	2	......	......	
Fatty degeneration	1	13	3	
5. *Diseases of Alimentary Tract.*				
Stomatitis	1	2	......	
Gastritis	4	1	2	
Gastric ulcer	11	1	1	10.
Gastro-enteritis	23	2	27	} 11.
Enteritis	57	139	31	
Colitis	15	......	1	
Acute tympanites	1	......	......	
Intussusception	1	......	......	
Obstruction	......	1	......	
Peritonitis	15	2	......	
6. *Diseases of the Urinary and Reproductive Organs.*				
Nephritis	4	1	......	
Inflammation of oviduct	......	2	1	
7. *Various.*				
Senile changes	1	....	......	
Anæmia without ascertainable cause	5	2	3	12

Table II. shows the distribution of these diseases amongst the principal classes of Mammals.

TABLE II.—Distribution of diseases amongst the Mammals.

Disease.	*Primates.*	*Carnivora.*	*Rodentia.*	*Ungulata.*	*Edentata.*	*Marsupialia.*
Tuberculosis	35	3	14	2	...	5
Filaria	3	...	...	...	...	...
Trypanosomiasis	...	...	1	...	...	...
Pneumonia	9	10	14	6	2	6
Septicæmia	2	5	...	4	...	1
Pseudo-tuberculosis	...	...	3	...	...	...
Hydatids	1	...	...	...	...	...
Worms	1	2	...	...	...	...
Itch	...	...	2	1	...	...
Broncho-pneumonia	17	5	3	10	2	7
Congestion of lungs	13	5	5	3	...	3
Pericarditis	1	3	...	2	...	1
Hepatitis	...	1	1	...	...	...
Fatty degeneration of liver	...	...	1	...	...	...
Stomatitis	1	...	...	...	...	...
Gastritis	...	1	1	1	...	1
Gastric ulcer	3	2	2	...	...	4
Gastro-enteritis	2	8	3	3	...	7
Enteritis	12	11	9	13	2	10
Colitis	8	3	...	2	...	2
Acute tympanites	...	...	...	...	...	1
Intussusception	...	...	...	...	...	1
Peritonitis	1	1	3	7	...	3
Nephritis	2	2	...	...	...	...
Senile changes	...	...	...	1	...	...
Anæmia (cause unknown)	...	1	1	...	1	2

Since the lesions of tuberculosis and mycosis are somewhat similar in appearance, care has been taken to separate them accurately, and the following Table sets forth their distribution amongst the different classes of birds.

TABLE III.—Comparative Table of the incidence of Tuberculosis and Mycosis in the various classes of birds.

	Tuberculosis.	*Mycosis.*
Passeres	34	9
Picariæ	2	5
Psittaci	2	11
Anseres	4	8
Columbæ	12	3
Gallinæ	29	9
Striges	2	5
Laridæ	1	5
Struthiones	2	

The seasonal variations of the principal diseases are very much as might be expected, and there seems to be little of importance to record in this connection. Tubercle remains pretty constant all the year round; mycosis has been more prevalent during the second and fourth quarters; pneumonia and broncho-pneumonia were more prevalent during the first and fourth quarters; and diseases of the alimentary canal have been more marked in the latter half of the year.

There has fortunately been no epidemic amongst the animals during the year, and disinfection has been carried out after every case of infectious disease, so as to lessen, as far as possible, the risks of infection. It will be noticed that there has not been a single case of tumour, cancerous or otherwise, in the animals examined during the year.

Notes on the foregoing Tables.

The following notes refer to a few points of special interest in connection with the diseases mentioned in the Tables.

1. Since the beginning of last year all reptiles have been systematically examined, and tubercle has been found in 17 cases out of a total of 161; and in 5 snakes, tubercular deposits have been found in the lungs. Pulmonary tubercle in reptiles has not, I believe, been described before, and the first specimen found has been preserved in the Museum of the College of Surgeons. Tubercle found in reptiles has so far been all of the avian type, both microscopically and in cultures.

The 88 cases of tubercle in birds seems a large number, but it compares favourably with the number of cases recorded* as occurring in the Berlin Zoological Gardens between December 1903 and August 1905. Between these dates 459 birds were examined there, of which 118 were tuberculous; our figures show that of 479 birds examined only 88 were tuberculous. In two parrots examined the tubercle was of the human type, in all the rest it was distinctly avian.

Of the 35 cases of tubercle in monkeys nearly all were of the human variety, two, however, were of a distinctly bovine type, and in one case the bacilli were of the avian type.

2. In four of the cases of mycosis the disease was due to *Aspergillus niger*, the lesions being similar to those produced by the *Aspergillus fumigatus* which was the organism associated with the remaining 51 cases.

3. Under the general term Malaria I have grouped a series of cases which, so far as I am at present able to state, are caused by parasites belonging to either the group of *Halteridium* or of *Proteosoma*. In these birds the parasites were certainly the cause of death, but they were also found in 12 other birds which died from other causes.

4. Several of the filaria found have not been described before and are still under investigation. In some cases two quite different kinds of filaria have been found in the same host, as in

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* Virchow's Archiv, vol. 90.

two Lawes' Birds of Paradise. The difficulty of identification is much increased by the fact that the parent worms often cannot be found. The filaria found in a Bearded Lizard is, I believe, the first found in a reptile.

5. A hitherto undescribed trypanosome has been found in a Francolin which died from mycosis, which is larger than any described so far in birds.

6. Hæmogregarines have been found in 40 reptiles, and in 13 of these, I believe, for the first time. In the cases in which death has been attributed to them the anæmia and blood destruction have been so extreme as to make the diagnosis quite certain; sometimes 50 or even 60 per cent. of the erythrocytes have been affected.

7. Pneumonia in reptiles is of two kinds—one a specific inflammation of the lung, the other a traumatic inflammation due to the irritation produced by ascaris eggs and embryos deposited there. In some cases masses like tubercles are formed around the source of irritation. 13 of these cases were of this irritative kind.

8. This protozoal infection occurred in a bull-frog, and the lesions were identical with those described by Danielewsky in 1889 in his account of the only other case on record in an edible frog. The causative organism which was present in the blood and the exudations in this case was a *Hexamitus* (really *Octomitus*).

9. Of the 29 mammals which died from congestion of lungs, 22 were suffering from rickets, 9 very severely.

10. It will be seen that gastric ulceration is more widely distributed amongst mammals than is usually thought to be the case.

11. The large number of cases of enteritis has led to an investigation into the probable causes. There appear to be three different kinds of enteritis in the Gardens—one which is caused by bacteria, one which is caused by protozoal organisms, and a third variety probably due to errors in feeding. An effort is being made to separate the various kinds, so that at any rate those due to feeding may be got rid of. The very large percentage of cases of gastro-enteritis in the reptiles will be noticed; in 58 out of 161 cases death was due directly to this condition, and in the remainder, which died of other diseases, the same condition was present in varying degrees. It would seem worth while to consider whether the present unnatural and unphysiological method of feeding the snakes has any connection with the very large incidence of inflammations of the alimentary tract occurring in them.

12. In these 10 cases there was very profound anæmia, with considerable blood changes. I think that these cases were in all probability parasitic in origin, but no cause, parasitic or other, could be found.

Several probably undescribed intestinal worms have been found during the year, and are still undergoing investigation.

The work condensed in this paper has been carried out in the laboratories at the Gardens and at the Lister Institute.

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FURTHER RESULTS OF THE EXPERIMENTAL TREATMENT OF TRYPANOSOMIASIS: BEING A PROGRESS REPORT TO A COMMITTEE OF THE ROYAL SOCIETY.

By H. G. PLIMMER, F.L.S., and W. B. FRY, Captain R.A.M.C.

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Further Results of the Experimental Treatment of Trypanosomiasis: being a Progress Report to a Committee of the Royal Society.

By H. G. PLIMMER, F.L.S., and W. B. FRY, Captain R.A.M.C.

(Communicated by J. Rose Bradford, M.D., Sec. R.S. Received June 28, 1909.)

The following results are a continuation of the work of which summaries have already appeared in the 'Proceedings of the Royal Society.'*

These experiments have been carried out, with the same strain of Surra as was used before, at the Brown Institution and the Lister Institute.

A.—*Condition of the Animals living at the Date of the Completion of the Tables in the last Paper.*

Rats treated with Sodium Antimonyl Tartrate, 1 *per cent.* (*p.* 478).

No. 7 died 428 days after inoculation.
„ 32 „ 409 „
„ 35 „ 371 „

Rats treated with Sodium Antimonyl Tartrate, 5 *per cent., in Colonel Lambkin's Medium* (*p.* 482).

No. 13 died 216 days after inoculation.

Rats treated with Antimony (*metal*), 5 *per cent., in Colonel Lambkin's Medium* (*p.* 483).

No. 10 died 205 days after inoculation.
„ 17 „ 367 „
„ 25 „ 385 „
„ 27 „ 399 „
„ 29 „ 360 „

Rats treated with Lithium Antimonyl Tartrate, 0·25 *per cent.* (*p.* 485).

No. 4 died 145 days after inoculation.
„ 5 „ 229 „
„ 6 „ 257 „
„ 8 „ 241 „
„ 10 „ 209 „

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* B, vol. 79, 1907, pp. 500—516; and B, vol. 80, 1908, pp. 1—12 and 477—487.

Most of the above rats died from cold; none of them died from the disease, and no trypanosomes were found in their blood or organs, and inoculations made therefrom were entirely negative.

B.—*Further Experiments.*

Rats treated with Lithium Antimonyl Tartrate, 1 *per cent.*

A further series of experiments has been carried out with this substance on a large number of rats, giving four doses of 4 to 5 minims (according to weight) of a 1 per cent. solution subcutaneously, a dose being given every other day. Practically by this method every rat can be cured. They have lived for varying periods, up to 249 days, and in no case have trypanosomes been found after death in the blood or in the organs. No rat has died of the disease, and in no case thus treated has there been a recurrence. The results have therefore been more constant than those attained with sodium or potassium antimonyl tartrates. The treatment was begun on the third or fourth day after inoculation; it will be seen below that when it is left until the number of trypanosomes in the peripheral blood is very great, although they may be driven out of the blood, it does not cure: so that the time at which treatment is commenced is of considerable importance.

It has also been given intravenously in rabbits, but with far less effect than when given subcutaneously. The elimination in this case is very rapid, to which fact we attribute its comparatively feeble action.

Rats treated with Lithium Antimonyl Tartrate on the Fifth or Sixth Day of the Disease.

The blood at this period of the disease is swarming with trypanosomes, and experiments were made in order to see what effect this salt of antimony would have upon the disease at this period. If one dose of 5 minims of a 1 per cent. solution be given the rats die on the seventh day, so that little or no effect is produced. If two such doses be given, one on the fifth and one on the sixth day, the average time of death in 10 rats was 19½ days, and living trypanosomes were found in the blood at death. When four doses were given, one on each day from the fifth to the eighth, the time in three rats was lengthened to 81 to 86 days: in one of these even living trypanosomes were found in the blood after death. By comparing these results with those mentioned in the former section it will be seen that the time at which the administration of the drug is begun is of importance, as well as the number of doses. The animals stand the best chance of cure when no recurrences take place, and this is best ensured by the method described in the previous section.

Further experiments made with Rats treated with Antimony in order to find out in what Organs the Trypanosomes are latent.

Following on the experiments made on rats treated with sodium antimonyl tartrate, with the view of finding out where the trypanosomes are latent, and recorded in the last paper,* a further series of experiments has been made on rats inoculated with Surra, which is more amenable to treatment with antimony than the Nagana used in the former series, and completely treated (that is, given a curative series of doses) with lithium antimonyl tartrate: this, as stated in the paper referred to, appears to be the most active of this variety of salt.

Seven rats were treated with four doses of 5 minims of a 1 per cent. solution of lithium antimonyl tartrate, and they were killed in succession, one on the 6th, 7th, 10th, 14th, 16th, 22nd, and 30th days after the last dose. The livers and bone-marrows were made into an emulsion with the minimum quantity of 0·89 per cent. salt solution, and 1 c.c. of the emulsions of these organs and 1 c.c. of heart's blood was injected separately into other rats. The results were entirely negative. Microscopic preparations were made of the material injected and no organisms were seen, and none of the sub-inoculations gave a positive result.

Experiments made in order to see if any Protection was afforded by Initial Treatment with Antimony.

A series of six rats was treated with four doses of 5 minims of a 1 per cent. solution of lithium antimonyl tartrate, one dose every other day in the same manner as when given for curative purposes. They were then inoculated with Surra, one on the first day after the completion of the treatment, and one on the 2nd, 4th, 5th, 9th, and 10th days after. They all died on the 5th or 6th days after inoculation, just as untreated rats would have done, so that antimony in this very soluble form is of no protective use in rats, owing most probably to its rapid elimination.

The blood of an uninfected rat treated as above has also been used in the *in vitro* experiments recorded below.

Rats treated with Sodium Antimony Lactate and with Antimony Sodium Calcium Lactate.

Through the kindness of Messrs. von Heyden we have been enabled to make some experiments with the above compounds. The sodium antimony lactate contains 26 per cent. of antimony, and the antimony sodium calcium lactate 17 per cent., so they are both much weaker in antimony than the tartrates which we have used. By the addition of a small quantity of lactic acid we were able to get a 1 per cent. solution of both salts, and in this strength the solutions were not very irritating, but neither with rats nor with larger animals are they as effective as the tartrates or the metal.

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* 'Roy. Soc. Proc.,' B, vol. 80, p. 487.

The following table shows the results obtained with sodium antimony lactate 1 per cent.

Average duration of untreated disease 6·9 days :—

Rats of 150 to 200 grammes weight.	Number of doses, and quantity.	Recurrences.	Lived.	Remarks.
1	2 of 4 minims	0	9 days	Died from enteritis.
2	4 of 4 ,,	0	20 ,,	Died from retained fœtus.
3	4 of 4 ,,	1	37 ,,	
4	5 of 4 ,,	1	46 ,,	
5	6 of 4 ,,	2	100 ,,	(No trypanosomes found in any of these rats after death.)
6	4 of 5 ,,	0	74 ,,	
7	5 of 5 ,,	1	48 ,,	

The following table shows the results obtained with antimony sodium calcium lactate 1 per cent.

Average duration of untreated disease 6·9 days :—

Rats of 150 to 200 grammes weight.	Number of doses, and quantity.	Recurrences.	Lived.	Remarks.
1	3 of 4 minims	1	83 days	No trypanosomes found *post mortem.*
2	4 of 4 ,,	0	25 ,,	Living trypanosomes found *post mortem.*
3	5 of 4 ,,	2	68 ,,	No trypanosomes found *post mortem.*
4	6 of 4 ,,	2	45 ,,	,, ,, ,,
5	5 of 5 ,,	2	64 ,,	,, ,, ,,
6	6 of 5 ,,	2	68 ,,	,, ,, ,,
7	8 of 5 ,,	2	57 ,,	,, ,, ,,
8	4 of 7 ,,	0	131 ,,	,, ,, ,,

On dogs the effect was very much less marked than on rats, and an effective dose became inconveniently large.

The following experiments show the relatively greater time taken for these salts to act, as compared with the sodium or lithium antimonyl tartrates, which drive all the trypanosomes from the peripheral blood in about an hour after the dose.

A Surra rat was taken on the fourth day, when the trypanosomes are numerous in the blood, and 5 minims of a 1 per cent. solution of sodium antimony lactate were injected.

Blood was taken	and showed the following :
½ hour after injection...	Trypanosomes affected by the drug: are extremely active, and show a tendency to swell.
1 " " ...	Very few normal trypanosomes to be seen: nearly all are swollen and spherical in shape (= "battledores"). Still large numbers.
1½ hours " ...	Much smaller number of trypanosomes to be seen: a few "battledores": a few motionless ones, and one or two normal forms.
2 " " ...	"Battledores" have all disappeared: one or two slowly moving normal forms seen.
2½ " " ...	Ditto.
3½ " " ...	No trypanosomes found.

A similar experiment made with a rat treated with antimony sodium calcium lactate yielded practically the same result. Further experiments made with these drugs *in vitro* will be mentioned later.

Experiments made with Antimony (Metal in state of finest Division) suspended in various Oily Media.

Since the curative results following treatment with the metal antimony* suspended in Colonel Lambkin's medium seemed promising, many trials have been made with the metal suspended in other oily media, such as olive oil, cod liver oil, lanolin, egg-yolk, etc., in order, if possible, to obviate, or at any rate reduce, the extremely irritating properties of the metal, which seriously interfere with its practical use.

In olive oil a 5 per cent. suspension was used: with one dose of 3 minims Surra rats lived for 15 days, and died with living trypanosomes in their blood. Seventeen Surra rats were given one dose of 5 minims on the fourth day of the disease, and they lived from 41 to 133 days: in these there were no recurrences, nor were trypanosomes found after death, and sub-inoculations were in every case negative. Six Surra rats were treated with the same dose in order to observe the time taken for the complete disappearance of the trypanosomes from the blood.

Blood was taken	and showed the following:
½ hour after injection...	Trypanosomes very active.
1 " " ...	As numerous: show evidences of swelling.
1½ hours " ...	Still numerous: nearly all swollen: some "battledores."
2 " " ...	Very few forms found: all "battledores."
2½ " " ...	No trypanosomes seen.

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* 'Roy. Soc. Proc.,' B, vol. 80, p. 483.

Two Surra rats were taken on the fifth day, when the blood was swarming with trypanosomes, and 6 minims were given. Two and a-half hours after the rats were killed, and smears were made from the lungs, liver, spleen, kidney, bone-marrow, heart's blood, and brain. In none of the specimens could a trypanosome be found after prolonged examination.

This oil was also given to several rats upon recurrences after treatment with small doses of the lactates mentioned above: in these cases the effect was much less marked, even although the number of trypanosomes in the blood was much less than in the rats treated for the first time. This accords with our general experience that recurrences are much more difficult to deal with than the initial infection, and this applies to all the drugs we have tried.

A suspension in cod liver oil took four hours to drive the trypanosomes out of the peripheral blood.

The suspension in egg-yolk appeared to act in rats better than any other; in dogs, however, the results were variable; sometimes strikingly good, at others no better than the other mixtures: sometimes causing great irritation and sloughing, sometimes not causing any irritation at all. We have rats alive for more than 120 days after inoculation, with no recurrences, after one dose.

An experiment was made to see how long one dose took to drive the trypanosomes out of the blood. A Surra rat on the fourth day was treated with 5 minims of a 5 per cent. suspension.

Blood was taken	and showed the following:
¾ hour after injection...	Trypanosomes much affected, but not decreased. Many "battledore" forms.
1¼ hours ,, ...	Trypanosomes reduced in numbers: all swollen and "battledore" forms: very little movement.
2½ ,, ,, ...	No trypanosomes found.

Experiments with Quassia.

Dr. Guillemard, of Cambridge, suggested that quassia, on account of its known poisonous effects on some of the lower forms of life, should be tested for its trypanocidal qualities. A series of experiments was therefore undertaken on rats.

Six Surra rats were treated on the third and following days of the disease with a 5 per cent. solution of the pharmacopœal extract of quassia: they were given three doses subcutaneously—5 minims on the third day, 10 minims on the fourth, and 10 minims on the fifth day. The trypanosomes were entirely unaffected, and the animals died on the sixth—seventh day.

Another series of 12 Surra rats was treated with a two hours' decoction of quassia-wood made with the minimum amount of water. Of this three doses were given—5 minims on the third day and 10 minims on the fourth and fifth days. The trypanosomes in these rats were also entirely unaffected, and the animals died on the sixth—seventh day. It was also tried intravenously in rabbits in doses of 30 minims of the decoction: no effect was produced, and the rabbits died on or about the 42nd day.

Experiments made *in vitro* correspond with these results, and will be described later.

Experiments with Arsenophenylglycin.

Professor Ehrlich kindly sent some of this substance to Dr. Bagshawe, the Director of the Sleeping Sickness Bureau, with which we have made some initial experiments upon rats. Ehrlich found that Nagana mice could be cured, in practically every case, with this substance. But the effects on larger animals, so far as we have gone, are not quite so satisfactory, and it compares in this undesirable manner very well with the antimony tartrates, with which we can cure practically every case of Surra in rats, but which do not have anything like the corresponding effects on rabbits, guinea-pigs, and dogs. It is not only in the question of practical dosage that difficulties arise: each kind of animal has a personal equation, and their reaction to a given drug is not similar. This, and the relatively larger dosage in bigger animals, present considerable practical difficulties in the treatment of trypanosomiasis.

Our experiments have given the following results. Out of eight Surra rats of 180 to 200 grammes weight which were given one dose of 25 minims of a 1 in 80 solution of arsenophenylglycin, four died on the 19th day with living trypanosomes in their blood, the recurrences having taken place on the 16th—17th day. Two were given three and five doses respectively of 5 minims of a 1 per cent. solution of lithium antimonyl tartrate on the 17th and following days, and they lived 59 and 51 days. Of the two which are still living (95 days), one has had five doses of 5 minims of a 1 per cent. solution of lithium antimonyl tartrate, beginning on the 17th day, and the other had one similar dose given on the day before the recurrences occurred in the other rats.

The following experiment shows the effect of this substance upon the trypanosomes in the blood, and how much longer it takes than the antimony salts to produce its effects.

A Surra rat on the fourth day of the disease was treated with 1 c.c. of a 2 per cent. solution of arsenophenylglycin (practically the same dose as given to the other rats).

Blood was taken	and showed the following :
½ hour after injection...	Trypanosomes showed slight increase of motility.
1 „ „ ...	Ditto.
2 hours „ ...	Ditto, but more marked.
3 „ „ ...	Trypanosomes not quite so active and fewer in number.
4 „ „ ...	Trypanosomes now very few in number.
4½ „ „ ...	Only one or two trypanosomes to be seen in a preparation.
5 „ „ ...	No trypanosomes seen.

In these specimens no swollen, breaking up, or "battledore" forms were seen : the trypanosomes simply disappeared.

On the Effects of the Drugs used upon the Trypanosomes in the Living Body.

In studying the therapeutic effect of the various drugs tried, including metallic antimony in a state of finest division, repeated observations of the peripheral blood were made in order to observe the effect of the drug upon the trypanosomes, and to ascertain when the trypanosomes entirely disappeared from the blood. The first stage noticed of the effect of the drug was a great increase in the motility of the trypanosomes, followed by a gradual slowing down to movements slower than normal. At this stage there is a tendency for the whole trypanosome to swell, and to become bloated in appearance. The swelling of the trypanosome continues until it becomes almost spherical in form, or oftener "battledore" shaped ; the protoplasm becomes indistinct, and the flagellum appears to be attached to only one side of the periphery ; the macro-nucleus is fairly distinct, but it eventually breaks up, and then the swollen mass disintegrates. The spleen at this time is full of these broken up masses of trypanosomes, and as the nuclei will still stain, a plasmodial appearance is seen in films of bits of nuclei dotted about in a granular ground. These stages can be observed after treatment with all the salts of antimony used, and are well marked after the administration of the metal, in which case, however, the stages are slower. The soluble salts, lithium and sodium antimonyl tartrates, effect the total disappearance of the trypanosomes in about one hour. Metallic antimony, when given in the various media tried (Lambkin's medium, olive oil, cod liver oil, heavy paraffin oil, egg-yolk), brings about this disappearance in from two-and-a-half to four hours, according to the medium used : the first noticeable effects being produced in about half an hour. In the case of egg-yolk and olive oil the blood is free from trypanosomes in two-and-a-half hours. This would seem to show that some portion of the metal introduced must be changed into some soluble form very rapidly; but apparently after the reaction of the tissues occurs the antimony becomes more or less shut off, and absorption must take place very slowly, as traces of the metal, apparently unaltered, have been found as late as six to seven weeks after the injection.

Sodium antimony lactate and antimony sodium calcium lactate were found to act rather more slowly than the above (see Table above), the time at which the trypanosomes had completely disappeared varying from three to four hours.

It was noticed in these experiments that trypanosomes, though obviously drug-affected when the blood was taken, remained alive on the slide outside the body for a long time after all forms had disappeared from the circulating blood.

Further details of the time taken for the various drugs to act will be found in the sections upon sodium antimony lactate, antimony oil, antimony egg-yolk, and arsenophenylglycin.

On the Action of Trypanocidal Substances in vitro.

Experiments have been carried out with a view of throwing light on the more exact nature of the changes which are produced in trypanosomes when they are brought into contact with trypanocidal substances. The general principles we have observed in these experiments have been: 1. To dissolve the drug in some fluid so that when it is added to the infected blood it will not cause osmosis to occur in the cellular elements of, or trypanosomes contained in the blood. (The various substances were dissolved in a 0·89 per cent. salt solution, isotonic with rat's blood which was used in these experiments.) 2. To use always equal volumes of the solution and of the affected blood. 3. To use blood at the time when the trypanosomes are just becoming very numerous, so as to avoid the presence of old, feebly moving forms, which are always present in the later stages of an acute infection. The method of observation has been to watch the behaviour of the trypanosomes when in contact with the various solutions of the drug under the microscope. A measured drop of blood and of the solution are mixed on a slide with care: the mixed drop is then covered with a sufficiently large cover glass, and this is sealed with vaseline.

It has been found possible in this manner to exactly determine the dilutions at which the various drugs used cease to have an instantaneously trypanocidal action; further, in higher dilutions, by carefully watching the changes taking place in the trypanosomes, it is possible to determine the dilution at which no effect is produced, and between these two points the periods of time necessary to ensure immobility and death of the trypanosomes can be ascertained. By a comparison of the results obtained a very good estimate of the probable action of any drug when given to an affected animal can be arrived at.

For instance, sodium and lithium antimonyl tartrates were found to act, in the same dilutions, in a manner fairly comparable to their antimony content, and to their action on the trypanosomes in an affected animal. Again, with atoxyl a much higher concentration of the drug was necessary—it had to be about ten times stronger—in order to obtain the same destruction pictures, results corresponding with the rapidity of the disappearance of trypanosomes from the peripheral blood of affected animals when treated with the above drugs.

In the case of the two new lactates mentioned above, their therapeutical value was accurately foretold by a preliminary study of their action *in vitro* in the manner described. In all these experiments controls have been carried out: it has been found that trypanosomes will live and retain their activity for hours when infected blood and the diluting fluid alone are mixed together.

The various changes taking place in trypanosomes on coming into contact with a dilute trypanocidal drug, commencing with their preliminary extraordinary increase of activity, and their subsequent swelling up, immobility, and disintegration, can be watched in all their different stages in this manner. These effects resemble very closely the changes which take place in the trypanosomes in the peripheral circulation of an animal treated with antimony.

The following tables show the effects produced by the different substances in their various dilutions.

Dilutions of sodium antimonyl tartrate in 0·89 per cent. salt solution mixed with Surra rat's blood, in equal parts. The control in all cases is equal parts of blood and 0·89 per cent. salt solution.

Dilutions.				Time.	Control.
1—500.	1—1000.	1—5000.	1—10,000.		
Motionless	Motionless	Few active forms	Trypanosomes active	1 min.	Very active.
,,	,,	Motionless	Few active forms; rest sluggish	10 mins.	,,
,,	,,	,,	All sluggish	30 ,,	,,
,,	,,	,,	Motionless	1 hour	,,

Dilutions of lithium antimonyl tartrate in 0·89 per cent. salt solution mixed with Surra rat's blood, in equal parts.

Dilutions.					Time.	Control.
1—500.	1—1000.	1—5000.	1—10,000.	1—20,000.		
Motionless	Motionless	Some active trypanosomes	Active trypanosomes	Very active trypanosomes	1 min.	Very active.
,,	,,	Motionless	Some active trypanosomes	Many active trypanosomes	10 mins.	,,
,,	,,	,,	Practically no motile trypanosomes seen, only 1 or 2 in a slide. Tendency to clump	Few active trypanosomes seen. Tendency to clump	30 ,,	,,
,,	,,	,,	Motionless	1 or 2 active forms seen. Rest motionless	1 hour	,,

In a dilution experiment with lithium antimonyl tartrate made with the blood of a Surra rat after a second recurrence, after treatment with antimony (metal) and on first recurrence with lithium antimonyl tartrate, the trypanosomes *in vitro* appeared to have a greater resistance to the dilute drug than the stock strain.

A comparison of the following table with the previous one will demonstrate this :—

Dilutions.		Time.
1—1000.	1—5000.	
A few active forms present.........	A number of active forms present	1 min.
Motionless	A few active forms seen......................	10 mins.
,, ...	Motionless	30 ,,

This bears out our experience that the recurrences become less and less amenable to antimony as they increase in number.

The following table shows the action of atoxyl and lithium antimonyl tartrate compared in the above manner :—

Dilutions of atoxyl.			Time.	Dilutions of lithium antimonyl tartrate.		
1—500.	1—1000.	1—5000.		1—500.	1—5000.	1—10,000.
Trypanosomes, all active	Active	Active	1 min.	Trypanosomes, all motionless	All markedly affected	All fairly active.
Active but affected	,,	,,	5 mins.	Motionless : commencing disintegration	Motionless	Less active.
Less active	Sluggish, but still many active	,,	15 ,,	Only *débris* seen	,,	Some still moving; tendency to clump.
Practically motionless	Nearly all motionless; 1 or 2 active forms seen	Many moving still	2 hrs.	,,	Disintegrated	Motionless; some disintegration.

Concentrated decoction of quassia in 0·89 per cent. salt solution mixed with Surra rat's blood in equal parts.

Dilutions.				Time.	Control.
1—500.	1—1000.	1—5000.	1—10,000.		
Very active	Very active	Very active	Very active	1 min.	Very active.
,,	,,	,,	,,	10 mins.	,,
,,	,,	,,	,,	30 ,,	,,
,,	,,	,,	,,	1 hour	,,
Less active	Less active	Less active	Less active	2 hours	Less active.

The conditions of the dilutions and the control were precisely similar at the end of two hours. There was no swelling nor clumping.

Dilutions of arsenophenylglycin in 0·89 per cent. salt solution mixed with Surra rat's blood in equal parts.

Dilutions.					Time.	Control.
1—100.	1—500.	1—1000.	1—5000.	1—10,000.		
Very active	Very active	Very active	Very active	Very active	1 min.	Very active.
Irritated : movements rapid and convulsive	Activity increased	,,	,,	,,	10 mins.	,,
Nearly motionless	Sluggish	,,	,,	,,	30 ,,	,,
Motionless	,,	,,	,,	,,	1 hour	,,

Experiments in vitro *performed with the Blood of a Normal Rat which had been treated with Antimony.*

Experiments were made in order to ascertain whether the blood of a rat which had been treated with antimony would show any active trypanocidal powers *in vitro.* Although in the case of an infected animal all the trypanosomes in the peripheral blood would have been destroyed in about an hour, no noticeable trypanocidal effects were shown by the blood of a treated rat in the following experiments.

A normal rat had 5 minims of a 1 per cent. solution of lithium antimonyl tartrate injected subcutaneously : its blood was taken at 15, 30, 60, and 70 minutes after the injection, and was mixed with an equal quantity of blood from a Surra rat containing many trypanosomes; the mixed bloods, taken at the times mentioned, were examined under the microscope at various intervals from 5 to 30 minutes after the mixing, and the trypanosomes were found to be entirely unaffected, so that the blood of the treated normal rat did not have any trypanocidal effect added to it by the dose of lithium antimonyl tartrate. The Surra rat, whose blood was used for this experiment, was then given 5 minims of a 1 per cent. solution of lithium antimonyl tartrate :—

Blood was taken	and showed the following :
10 minutes after injection...	Trypanosomes affected : movement very rapid.
20 „ „ ...	Many "battledores."
40 „ „ ...	Trypanosomes greatly decreased in number all "battledores."
60 „ „ ...	Blood quite free from trypanosomes.

A normal rat was given four doses subcutaneously, one every other day, of 5 minims of a 1 per cent. solution of lithium antimonyl tartrate: 24 hours after the last dose a drop of its blood was mixed with a drop of blood from a Surra rat in which trypanosomes were plentiful. The mixture was watched under the microscope for half an hour, but no effect was produced: the blood of the treated animal behaving just as the blood of the control, an untreated rat.

A normal rat was given subcutaneously 10 minims (a lethal dose) of a 1 per cent. solution of lithium antimonyl tartrate, and its blood was mixed at half an hour, one hour, and one-and-a-half hours after the injection with an equal part of an emulsion of trypanosomes prepared from the lungs, liver, and heart's blood of a Surra rat just dead. Each of the mixtures was examined up to 30 minutes, but no effect whatever was produced on the trypanosomes. These experiments may be compared with those recorded on p. 356.

Experiments with Antimony upon Dogs.

Since the date of the last paper a large number of experiments have been made with antimony in various forms upon dogs suffering from Surra. Of the five dogs mentioned there one remains alive and well at the present date, more than a year after inoculation.

Our experiences with dogs show that they are extremely susceptible both to the disease and also to antimony: they are therefore not quite suitable animals for these experiments, although they have all lived many times the length of the untreated disease, that is 14 days. Five of the dogs were treated with small doses of sodium antimonyl tartrate in their drinking water, but the disease is so acute in dogs that this method of giving the drug, although it appeared to have some effect in postponing the reappearance of the trypanosomes in the blood, did not produce results sufficiently encouraging to warrant further experiments.

With regard to the experiments made with metallic antimony suspended in egg-yolk, the initial experiment was so encouraging as to make a further trial necessary. In this case the dog at the first relapse was given 20 minims of a $2\frac{1}{2}$ per cent. suspension: there was no local reaction, which in dogs is of frequent occurrence after the administration of antimony in any form, and the trypanosomes, which were very numerous, were entirely absent from the blood in 24 hours; the dog remained quite free from them for 48 days, and gained 3 lbs. in weight, and appeared perfectly well. The recurrence was very sudden, as the dog was perfectly well up to the moment when he was seized with a series of fits which ushered in the recurrence, from which he did not recover. A rat treated at the same time as this dog with 5 minims of the same suspension is alive and well more than 100 days after this one dose.

Many of the dogs mentioned in the table below have died with fits and paralyses and other nervous symptoms, but we are not certain whether these are due to the disease or to the antimony. In certain of the dogs the treatment has appeared to alter the acute disease into a chronic one, and in one of these more chronic cases there was a considerable excess of cerebro-spinal fluid and a cellular exudation around the vessels in the brain, very similar in incidence and extent to that described and figured by one of us in rats dead from infection with *Trypanosoma gambiense*.*

There is a curious uncertainty in the local effects produced in dogs by antimony, whether injected subcutaneously or intramuscularly, and they vary from time to time in the same dog; sometimes little or no effect is produced, and sometimes the suppuration and necrosis produced are sufficient to kill the animal.

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* 'Roy. Soc. Proc.,' B, vol. 79, p. 95.

We have recently given 24 injections of lithium antimonyl tartrate subcutaneously to three dogs in the greatest possible dilution: of these three places have suppurated slightly, although the conditions under which they were given were similar to those under which the 21 other doses were given. (These dogs are now living and well 53 days after inoculation, and they have had no recurrences.)

The following table gives a synopsis of the treatment, etc., of Surra dogs:—

Average Duration of Untreated Disease, 14 Days.

No.	Weight, in kilos.	Number of doses.	Quantity of dose, in minims.	Material.	Recurrences.	Remarks.
1	11	2	20	5 per cent. ant. cream	2	*Dog is alive and well 373 days after inoculation.*
		2	20	2 ,, sod. ant. tart.		
2	11	4	20	2 ,, ,, ,, ...	3	Died on 94th day : no trypanosomes found for 21 days before death. There were 41 days between the first and second recurrences. Died with fits and nervous symptoms.
		8	20	2 ,, lith. ant. tart.		
3	18¾	4	20	5 ,, sod. ant. tart. cream......	6	Died on 67th day : no trypanosomes found for 22 days before death. Died with fits and nervous symptoms.
		2	20	2 ,, ,, ,,		
		1	20	5 ,, ant. cream		
		4	20	2 ,, lith. ant. tart.		
4	8	3	12	5 ,, sod. ant. tart. cream......	4	Died of distemper on 63rd day. No trypanosomes found for 7 days before death.
		1	20	2 ,, ,, ,,		
		7	12	2 ,, lith. ,,		
5	6¾	3	10	5 ,, sod. ,, cream......	3	Died of pneumonia on the 53rd day. No trypanosomes found for 11 days before death.
		2	20	1 ,, lith. ,,		
		3	10	2 ,, ,, ,,		
6	6¼	5	10	2 ,, ,, ,, ...	1	Died from abscess on the 40th day. No trypanosomes found for 17 days before death.
		4	20	2 ,, ,, ,,		
7	7¼	5	10	2 ,, ,, ,, ...	3	Died from abscess on the 61st day. No trypanosomes in blood for 10 days before death.
		7	20	2 ,, ,, ,,		
8	14½	4	20	2 ,, ,, ,, ...	2	Died on 55th day with fits and nervous symptoms. Trypanosomes in blood. Antimony given in water also.
		2	12	5 ,, ant. oil		
9	12¾	2	15	2 ,, lith. ant. tart.	2	Died on 77th day from abscess. No trypanosomes seen for 16 days before death. Antimony given in water also.
		2	15	5 ,, ant. oil		
		1	20	5 ,, ,,		
10	13¼	2	12	5 ,, ,, ...	3	Died on 63rd day with nervous symptoms and paralysis. No trypanosomes found. Antimony given in water also.
		1	20	2 ,, lith. ant. tart.		
		2	15	2 ,, ,, ,,		
11	8½	3	20	1 ,, ant. sod. lact.	4	Died on the 66th day with nervous symptoms. No trypanosomes found after death. Antimony given in water also.
		1	15	2 ,, lith. ant. tart.		
		1	10	5 ,, ant. oil		
		1	15	5 ,, ,,		
		1	5	2½ ,, ,,		
		2	15	2½ ,, ,,		

Average Duration of Untreated Disease, 14 days.

No.	Weight, in kilos.	Number of doses.	Quantity of dose, in minims.	Material.	Recurrences.	Remarks.
12	10	1	20	1 per cent. ant. oil	3	Died on the 65th day with living trypanosomes in blood. Antimony given in water also.
		2	10	5 ,, ,,		
		2	15	5 ,, ,,		
		4	15	2 ,, lith. ant. tart.		
13	11½	1	20	2 ,, ,, ,,	1	Died on the 60th day with nervous symptoms. No trypanosomes seen for 29 days before death.
		1	15	2½ ,, ant. oil		
		2	15	5 ,, ,,		
		1	20	5 ,, ,,		
14	10½	2	15	5 ,, ,,	2	Died on the 52nd day from abscess. No trypanosomes found after death.
		1	20	5 ,, ,,		
		1	15	2 ,, lith. ant. tart.		
		2	20	2 ,, ,, ,,		
15	13¾	1	15	5 ,, ant. oil	5	Died on the 50th day with living trypanosomes in blood.
		2	20	5 ,, ,,		
		3	20	2 ,, lith. ant. tart.		
16	9¾	1	15	2 ,, ,, ,,	0	Died on the 64th day from abscess. No trypanosomes found after death and no recurrences.
		2	15	5 ,, ant. oil		
		1	10	2½ ,, egg ant.		
17	9¼	2	15	5 ,, ant. oil	0	Died on the 48th day from pneumonia. No trypanosomes found after death.
		1	15	2 ,, lith. ant. tart.		
18	9¼	1	15	2 ,, ,, ,,	1	Died on 50th day, possibly from ant. sod. lactate. No trypanosomes found after death.
		2	15	5 ,, ant. oil		
		1	20	1 ,, ant. sod. lact.		
19	10¼	1	20	2 ,, lith. ant. tart.	3	Died on 74th day with nervous symptoms. There were 48 days between the first and second recurrences.
		1	15	5 ,, ,, ,,		
		1	10	5 ,, ,, ,,		
		1	20	5 ,, ant. cream		
		1	20	2½ ,, egg ant.		
		1	10	2½ ,, ,,		
20	7¼	3	15	2½ ,, ,,	—	Trypanosomes practically never out of blood. Died on the 37th day, paralysed.
		1	15	5 ,, ,,		

21	13½	2	10	2½ per cent. egg ant.	—	Trypanosomes practically never out of blood. Died on the 44th day with fits and nervous symptoms.
		3	20	5 ,, ,,		
		1	15	2 ,, lith. ant. tart.		
22	8½	3	15	2½ ,, egg ant.	—	Trypanosomes practically never out of blood. Died on the 55th day with fits and nervous symptoms.
		3	15	2 ,, lith. ant. tart.		
23	13¼	1	15	5 ,, egg ant.	3	Died on the 55th day with fits and nervous symptoms.
		2	20	5 ,, ,,		
		2	15	5 ,, lith. ant. tart.		
		4	5	5 ,, ,, ,,		
24	12¼	1	15	5 ,, egg ant.	2	Died on the 64th day with living trypanosomes in the blood.
		1	20	5 ,, ,,		
		1	15	5 ,, lith. ant. tart.		
		1	10	5 ,, ,, ,,		
		5	5	5 ,, ,, ,,		
25	9¾	1	10	5 ,, ,, ,,	2	Died on the 56th day with nervous symptoms.
		1	20	5 ,, egg ant.		
		5	5	5 ,, lith. ant. tart.		
26	8	1	10	5 ,, ,, ,,	2	Died on the 47th day. Trypanosomes found in the cerebro-spinal fluid.
		1	20	5 ,, egg ant.		
		5	5	5 ,, lith. ant. tart.		

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Studies on Ceylon Hæmatozoa.

No. 1.—The Life Cycle of Trypanosoma vittatæ.

By
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Carnegie Fellow in the University of Glasgow.

With Plates 16 and 17 and 4 Text-figures.

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I. INTRODUCTION.

IN August, 1907, I went to Ceylon with a view to investigating the Protozoan blood parasites of Reptiles. The following memoir gives an account of the Trypanosome infection found in the soft Tortoise Emyda vittata.

I wish here to express my great indebtedness to Dr. Arthur Willey, F.R.S., Director of the Government Museum in Colombo. He not only obtained laboratory facilities for me at the museum but also gave me the most enthusiastic assistance in every possible direction. In some instances, at very considerable inconvenience to himself, he actually sent me the infected material which he had come across while engaged upon other work in the jungle. Indeed it is to him, as will appear later, that I owe the discovery of the true intermediate host in the infection which forms the subject of this paper.

Emyda vittata is a soft tortoise covered all over with soft skin, coloured black above and pure white underneath, hence its native name of "kirri ibba" or milk turtle.

The animal has flaps on the under side so arranged that all the four limbs and the head can be completely withdrawn. Like most of this group it is more or less nocturnal in its habit, it is said to leave the water very rarely, but I have myself, while watching a pool near Allutoya, where the country is all jungle, seen one come out of the water immediately after sunset and start prowling about at the edge. These soft tortoises die if they are kept out of water for more than a few hours, so that in order to carry them safely from one place to another it is necessary to wrap them in a damp cloth or put them in a wet bag of sacking or palm leaf. The creature is to be found practically all through the low country. I examined specimens from Colombo, from Kesbewa and Hanwella (both not far from Colombo) from Hambentot, which is on the eastern side, and from Trincomalee, also on the eastern side. It is, relatively speaking, common, but not nearly so abundant as the lake tortoise, Nicoria trijuga, as far as I could make out they were on the whole more plentiful on the eastern side than in the west.

I never heard of Emyda vittata being seen up country, although Nicoria trijuga is to be found in large numbers at Peradeniya and in the Kandyan district generally, which is at an elevation of nearly 1500 feet.

Emyda vittata is very generally infected both with a large Trypanosome (Plate 16, figs. 1—4), and with a Hæmogregarine. I never came across a really satisfactory negative case, although cases were observed where one or other of the parasites appeared to be absent. Protozoologists are quite familiar with the difficulty of being certain that a negative diagnosis is correct. I several times tested apparently negative specimens only to find that at last a stray parasite or two did finally turn up.

I attempted to obtain uninfected specimens by hatching out the eggs already provided with hard shells which were found in the oviduct of a freshly-captured Emyda. There were five eggs but none of them hatched out, and Mr. Fernando, the taxidermist of the museum, who had, he told me, frequently tried similar experiments, said that he had never got eggs obtained in this way to hatch out. It might possibly be that the length of time that the egg spent in the oviduct before being laid had some effect upon its capability for further development. One of the eggs was opened after more than three weeks, and although it was quite well preserved no development had taken place.

As a rule the Emyda was fairly well infected and in some cases the blood contained a very large number of parasites. I may say at the outset that in spite of the most diligent search and of a slight theoretical bias in favour of the hypothesis I could not find any connection between the Trypanosome and the Hæmogregarine infections. I propose to discuss the Hæmogregarine in a subsequent paper.

As far as my observation goes this Trypanosome is only found in Emyda vittata and I have called it Trypanosoma vittatæ. A Trypanosome is found in the fish Saccobranchus (see "Some Ceylon Hæmatozoa," Drs. Castellani and Willey, 'Quart. Journ. Micr. Sci.,' vol. 49, 1905), which inhabits similar localities, but this is obviously a quite distinct species. Nicoria trijuga, though often to be found living side by side with Emyda vittata, and also as in the Colombo lake in water which harboured a very large number of the generally infected Saccobranchus, never showed any sign of a trypanosome infection at all. My own observations upon blood parasites in Ceylon and those of many observers upon the European forms, especially those occurring in birds and amphibians, point towards the necessity of exercising considerable care before deciding that any hæmatozoon is specific to any given vertebrate host. Nevertheless, in the present instance I feel considerable confidence in attributing the Trypanosome in question exclusively to Emyda vittata.

Trypanosoma vittatæ is a large form resembling

T. raiæ in its external appearance and partly also in its movements in rather a remarkable manner, the most striking difference being in the situation of the trophonucleus which in T. vittatæ lies much nearer to the kinetonucleus.[1]

II. Observations upon the live Trypanosome.

A great deal of time was spent in making observations upon the living object, as it is obvious that where possible it is by far the most satisfactory method.

Trypanosoma vittatæ in the live state is a pyriform organism with a well developed frilled membrane. The frilled appearance is of course due to the membrane being longer from tip to tip at the free edge than at its origin from the protoplasmic body. The trophonucleus can be clearly distinguished as a circular body lying at no great distance in front of the kinetonucleus. It appears as a greyish sphere surrounded by a brighter halo: there is something very characteristic in the rather soft way in which the nuclear structures refract the light, contrasting sharply with the very hard, bright appearance of the protoplasmic inclusions—this is alike true of Trypanosomes and Hæmogregarines. Striations or myonemeta can be seen running longitudinally along the body; these are sometimes extraordinarily clear, and are especially conspicuous just before the animal rounds itself off.

---

[1] I have adopted the now very generally accepted terms of kinetonucleus and trophonucleus for the small and large nuclear bodies respectively. These terms seem to me to express more adequately than any of those hitherto proposed the nature and function of these two structures. In this paper the expression "anterior" end is used as equivalent to the flagellate end, "posterior" end as equivalent to non-flagellate end. The evidence in favour of this view being correct seems quite convincing when one has regard to those Trypanosomes in which the Trypanosome phase is derived from a Crithidial or Herpetomonad form in the normal life cycle. The evidence for regarding the flagellate end as the anterior is not so clearly indicated in Trypanosomes which adopt the crithidial or herpetomonad condition by the mere alteration in shape of the body and the migration towards the flagellate end of the already existing flagellum. This development, as is well known, is said to occur in the cultured forms of a very large number of different Trypanosomes, notably those of birds and mammals.

The flagellum runs forward from the kinetonucleus—which can occasionally be distinguished in the live specimen—along the edge of the undulating membrane and ends in a long free whip. The waves of contraction which pass along it make little sharp corners appear at what may be called the bays of the frills, giving a very characteristic appearance, though one difficult to describe in words.

Bright inclusions may be present all along the body, arranged without any appearance of regularity; they are often entirely absent, and I never discovered upon what either their absence or their presence depended. The body of the Trypanosome is apparently oval in section. The posterior end extends some way beyond the kinetonucleus; it is changeable in shape, and may be drawn out to a rapidly tapering point, or be rounded off and rather blunt.

The movements of this Trypanosome are rather complicated, and it can execute a considerable number of different figures. Like many Trypanosomes its motion of translation in the blood of the vertebrate host is relatively speaking slight; this is in marked contrast to the extraordinarily rapid darting movements of certain of the forms developed in the transmitting host. Quite possibly this lesser power of actual translation through space is correlated with the fact that the parasite in the vertebrate blood is in a medium which is itself in motion. Trypanosoma vittatæ shows the wheel motion so often seen in Trypanosomes in fishes; it also executes repeated serpentine twisting, sometimes in a figure 8, or even following a simple U-curve down one limb and up the other. The most characteristic movement, however, is a rather slow, forward spiral twisting. The Trypanosome will sometimes go on revolving slowly round its long axis with the body in the shape of a corkscrew, and with hardly any forward motion at all. The spiral twisting often occurs rhythmically forwards and backwards, through a distance of about twice or thrice the length of the Trypanosome. This last movement is familiar to observers who have worked with Spirochætes, only in these it is intensely rapid, while in the case of the Trypanosomes it is quite a slow movement.

In most infections small Trypanosomes (Pl. 16, figs. 5—7) are to be seen, less than half the size of the average specimens. In these the membrane is generally a little wider relatively than in the case of the larger forms, and the part of the body posterior to the kinetonucleus is much shorter and generally more pointed. The protoplasm is usually rather hyaline. I do not think that these small specimens belong to a separate species, as forms intermediate in size are also to be seen. Further, these small creatures take part equally in the developmental process to be described presently. I am, however, ignorant of their origin.

Another variation, only rarely met with, is that some specimens have the trophonucleus very much further forward than is usual. These creatures were present in small numbers only in one infection. The ordinary forms were also present. I cannot be certain as to whether they belong to the same species or not, but am personally inclined to think that they do.

There is always a considerable variation in the size and thickness of the Trypanosomes, and also, to a certain extent, in their staining reactions; but it is not marked enough for there to be any reason, in my opinion, for dividing them into male, female, and indifferent upon their morphological characters. This type of difference between the forms is much less evident than, for instance, in such a Trypanosome as T. brucei.

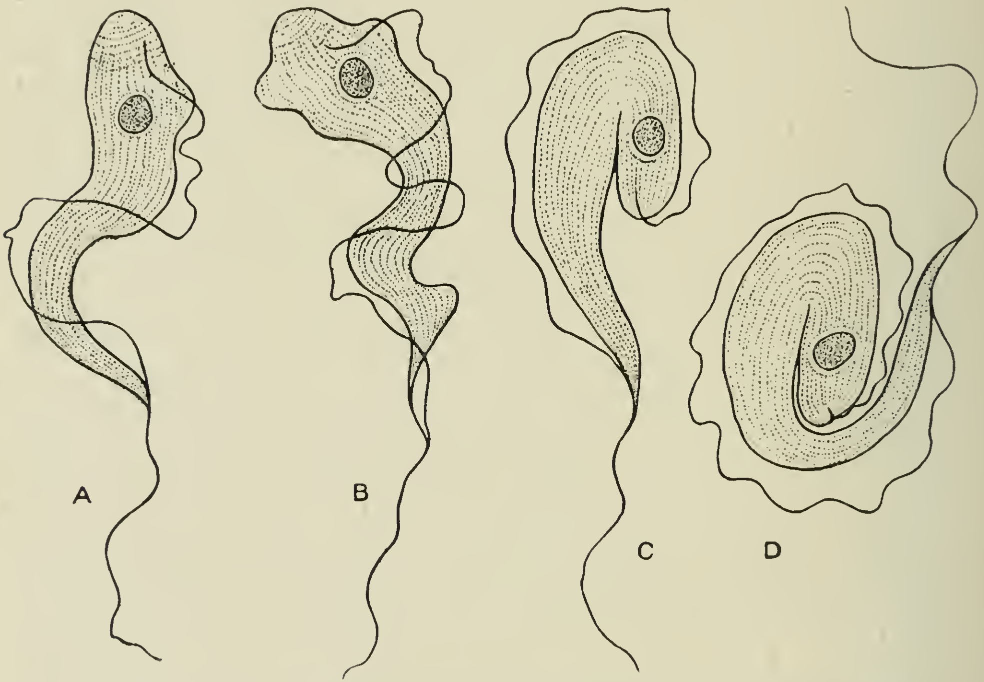
Longitudinal division does occur, but it is only very rarely to be seen, even in good infections. Specimens with two trophonuclei are very occasionally to be seen; it so happens that I have seen these chiefly among the intermediate sized forms.

If blood infected with Trypanosoma vittatæ is placed upon a slide, covered with a coverslip and sealed with vaseline, quite a different form of multiplication can be observed. The first time I observed this, a slide with blood very strongly infected with Hæmogregarines, and only relatively slightly with Trypanosomes, had been left overnight. Various alterations in the appearance of the Trypanosomes, to be described presently, had been noted before leaving the slide in the evening. Early next morning the slide was found to contain quite a number of small flagellates, just about the size of the Hæmogregarines and very much smaller than the Trypanosomes. A few unaltered Trypanosomes were present, but no intermediate forms. The appearance of the flagellates strongly suggested a connection with the Hæmogregarines. They had comparatively short flagella, the membrane reaching to about between one third and two thirds of the way up the protoplasmic body in different specimens. In fact, they showed a tantalising resemblance to the figure of the Trypanosome phase in the blood of the little owl, as described in Schaudinn's well known memoir, and attributed by him to the life-history of Proteosoma noctuæ.

The experiment was repeated several times, and the following development was made out, showing clearly that the organism arose from the Trypanosome.

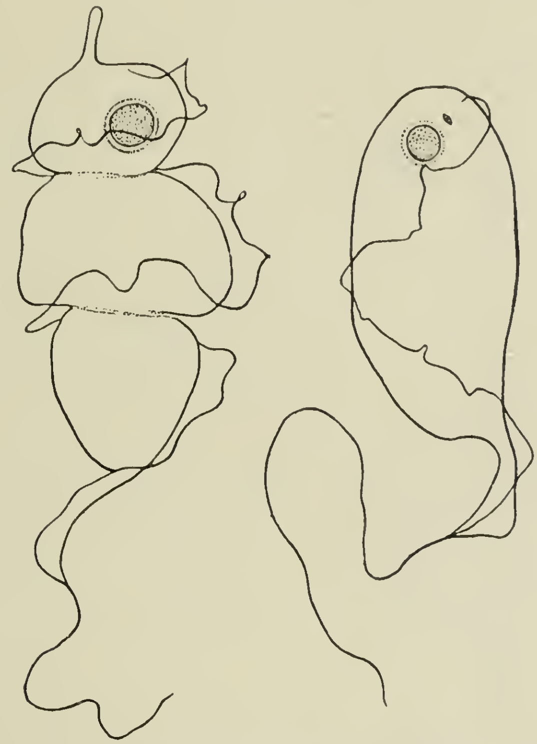
Some time after making the preparation the Trypanosomes begin to show various modifications in the external appearance. The length of time which elapses before the creature begins to yield to the altered conditions is remarkably variable, the time co-efficient throughout the whole process is in fact very inconstant. Generally speaking the organisms remain unaltered for about an hour and a half. The alterations in appearance culminate by the complete loss of the Trypanosome shape and the rounding off of the organism, but this condition is arrived at in various ways. Some of the Trypanosomes simply become much thickened at the non-flagellate end. Many become bent upon themselves, and the two limbs of the bend then fuse together (text-fig. 1, C and D). The text-figures illustrate these appearances. The myonemata become much more evident in most cases during these early phases. In some cases the animal broadens considerably and adopts the spiral shape, the turns of the spiral fuse together, and the most grotesque dumpy creature is produced, which keeps up a slow corkscrew or revolving motion (text-fig. 2).

Another appearance of rather a curious character is that where the screw movement backwards and forwards is kept up but very slowly, and the body no longer preserves its regular fusiform shape, but bulges now in one direction now in another (text-fig. 1, A and B). The movement is difficult to convey, but is best described as very metabolic euglenoid movement associated with a slow screwing backwards and forwards. During this movement the myonemeta can still be very clearly seen, and besides these at the non-flagellate end circumferential lines running round the creature can be seen, especially during the screw forward.



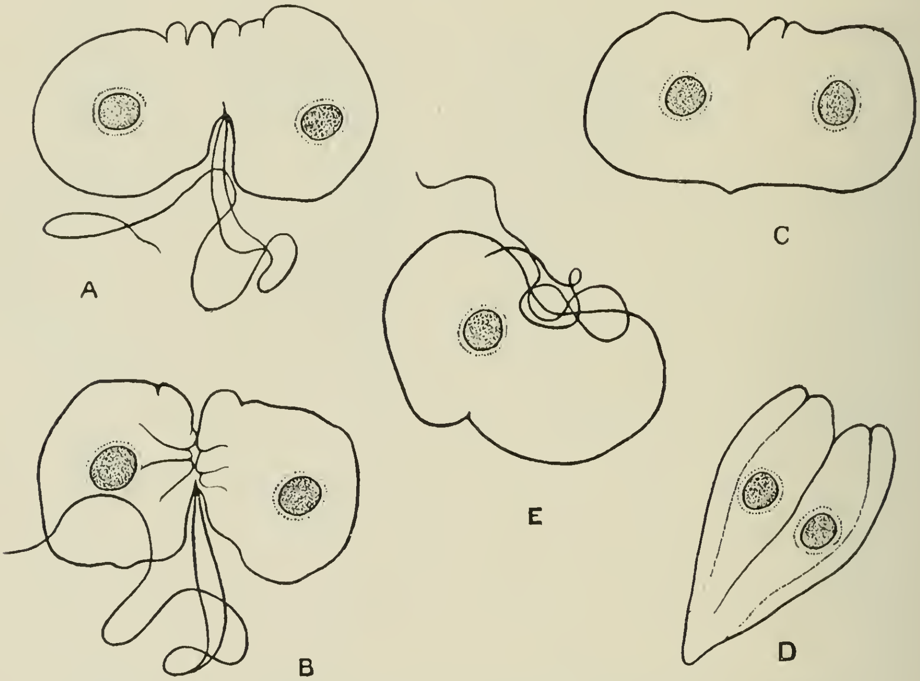
TEXT-FIG. 1.—Sketches of live Trypanosomes to show early phases in the rounding off of the parasites. A and B are one individual, so also C and D.

These appearances seem on the surface to show a curious amount of variation, but it is easily explained if it is remembered that at this stage an obvious decrease in the firmness of the peripheral protoplasm is taking place; in fact it becomes much more soft and viscid. This in correlation with the various methods of movement found in the normal trypanosome produces all the figures noted above. Thus, for instance, in text-fig. 1, C and D the Trypanosome has obviously been executing the wheel motion when its protoplasm began to soften, and fusion occurred, and so on.



TEXT-FIG. 2.—Two different methods of rounding off.

Finally, whatever the method adopted the Trypanosome comes to rest, and the flagellum breaks loose from the membrane while retaining its attachment at the kinetonucleus. It still lashes about for a time. All trace of the myonemeta completely disappear, and the animal appears as an irregular mass of protoplasm (text-fig. 3, E). Occasionally part of the body at the extreme anterior end (flagellate end) projects—this is, as far as I could make out, not withdrawn into the body, but seems like the membrane and flagellum to disintegrate. After a time the nucleus becomes quite indistinct, and I noticed that after this I was never able to get a very clear view of the nucleus until just before the flagellate condition was again adopted, when it showed with customary distinctness. Furrows now begin to appear in the animal, and it divides into two (text-fig. 3, A and B). Another division follows this, and four irregular rounded or pear-shaped creatures are thus formed, lying generally more or less connected. They now put out each a flagellum on one end. The flagellum is at first simply a short thick process. It lengthens and begins to lash slowly from side to side, but is as yet not capable of moving the body. Presently a slight oscillation of the body of the parasite is to be observed, and ultimately as the flagellum lengthens the creature becomes motile.



Text-fig. 3.—Stages in the crop of the leech. A and B. Division stage of one individual. C and D. Individual adopting the pear shape at time of division. E. Newly-rounded off Trypanosome with flagellum still attached.

On one occasion I observed the whole process under a high power in an already pear-shaped individual. The flagellum can only be said suddenly to have appeared as a short, relatively thick process at the blunt end of the organism. This lengthened and became motile, and after a time its origin from the body appeared to lie more laterally, and a slight ridge became visible at that point. I am inclined to think that the ridge is the first appearance of the undulating membrane.

At this stage again, both as observed on the slide and when the process takes place, as will be seen later, in the leech, much variation in small detail is to be remarked, especially in relation to the relative times at which the different processes occur. Thus in the present case the preparation for the second division may, and very often does, take place before the completion of the first. Or, on the other hand, the two products of the first division may become quite separate before any preparation for the second division can be detected. In the matter of the flagella there is also much variation. Sometimes all the four flagella are developed before the first division occurs, or this may not take place until the completion of the second division. Generally speaking, the development of the flagellum lags behind when the process occurs on the sealed slide, while in the leech the flagella are developed as a rule very early.

The typical pear shape, which ultimately becomes fusiform, may be adopted very early; in fact, sometimes at the second division the protoplasmic body will adopt the form of a longitudinally-furrowed cone rounded at the broad end. These furrows are rather curious, as there may be a number of them giving the animal a ridged appearance. The deepest furrow is where the ultimate line of division occurs. The other furrows disappear. The length of the flagellum is again in some cases considerable before the body of the organism begins to lengthen at all, and rounded little creatures, with quite long flagella, may not uncommonly be seen in blood from the crop of the leech; they are of quite rare appearance on the sealed slide of blood direct from the Emyda vittata.

At this stage of the investigation the question of the transmitting host came to be considered. This was the more difficult to determine as no parasites of any kind had been found on the milk tortoises. The aquatic habit of the tortoise pointed to some water inhabiting blood-sucking form, and of these the leeches seemed the most likely. A number of the common Ceylon water leech, called by the natives Diya kudella,[1] were investigated, but none of them showed any sign of the trypanosome. It was found, however, that the leech fed readily upon the tortoise. Upon puncturing the crop immediately after feeding it was seen that the Trypanosomes began to undergo the above-described transformation at once upon being taken into the crop.

Thus a leech was put on to a milk tortoise at 8.30 a.m.; at 10.30 it was still feeding; at 11.30 it was found free in the tank. The crop was punctured at once, and the blood examined in the usual way on a slide ringed with vaseline. The Trypanosomes were for the most part already casting off their flagella, and many of them had already undergone the first division. By 1 o'clock they were divided into four, and many of the resulting Herpetomonas forms had become free, and were swimming about on the slide. Blood was then taken from another part of the crop, and the state of the parasites was exactly similar to that on the sealed slide.

The puncturing of the leech does not apparently cause it much inconvenience. The specimen mentioned lived quite well till the next day, when I finally opened it at 7.30 a.m. Many actively motile forms in the Crithidial stage were to be seen.

Further divisions it seems may occur after the two mentioned, and also a secondary increase in size. Some of the individuals had become considerably lengthened, and were rather slender, but in almost every case the trophonucleus was posterior to the kinetonucleus.

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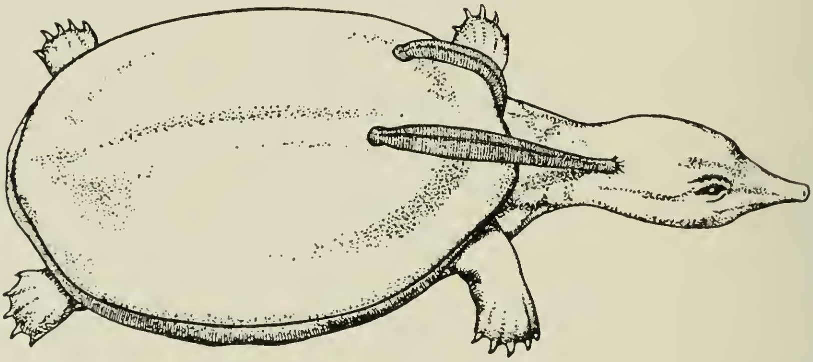
[1] Mr. W. A. Harding, who kindly examined the leeches brought from Ceylon, considers this leech to be Limnatis (Poecilobdella) granulosa.

Another leech killed ninety-five hours after feeding on the same tortoise showed slender Crithidial forms in the crop and some irregularly-shaped individuals. The intestine never showed many of the flagellates. A number of leeches were allowed to feed on various tortoises with the intention of observing the progress of the infection at different distances of time. Unfortunately, the work was at this point interrupted by illness for a number of weeks. On resuming it I found that the Trypanosomes still did persist in the crop in a few cases, but they were always very scarce. In one positive case they were to be found six weeks after feeding. An experiment was tried by feeding a leech for a second time on infected blood to see if the second infection would persist in greater numbers than the first, but by some fatality the tortoise ate the specimen that I had in this condition, and I never made out if the parasite showed any signs of becoming acclimatised to the host. The tortoise showed the greatest desire to eat the leeches, and had to be carefully watched; even so, it was surprising how often the more lively Emydas got the leech. They gulp them down, but do not break the skin with their teeth. The leech seems rather difficult to swallow, but the tortoise is most persevering, and it is almost impossible to rescue the leech once the tortoise has got well started.

I mention this, as there is always the possibility of parasites being spread by way of the digestive tract. The leech generally prefers to sit on the carapace attached by the posterior sucker and to fix its anterior sucker to the occiput, or back of the neck, or into the humeral angle. The accompanying text-figure is from a pencil sketch made in a few minutes by Dr. Willey, and gives a very typical picture. The leech will stretch to an almost incredible extent when the tortoise puts out its head rather than let go.

There is one point in the habits of this leech worthy of mention, namely, the fact that it will often feed in two instalments. Thus a leech would attach itself and feed for about an hour, and then cease and move about on the carapace, or even leave the tortoise altogether, and upon being replaced would take a second meal. I was in some doubt as to whether the leech had always fed the first time, but in a few cases it certainly had, and it had generally made the characteristic little wound. This habit, I think, may have a certain importance, owing to the very rapid changes which take place in the Trypanosome in the crop. I do not wish to imply that the horse-leech is likely to be a facultative transmitting host for this particular Trypanosome in nature, but the fact is of interest in regard to the transmission of parasites by leeches generally.



TEXT-FIG. 4.—Poecilobdella granulosum feeding on Emyda vittata. (Drawn by A. K. Maxwell from a sketch by Dr. A. Willey.)

The time taken to digest a meal appears to be much shorter in the Ceylon freshwater-leech than, for instance, in such a creature as Pontobdella muricata, the common marine leech which infests the skate of European seas, where very many months elapse before digestion is complete. Thus one of the Ceylon leeches which had fed vigorously so that it had been seen to swell out to a great extent, was nearly empty as regards its crop after fifty-three days. The size of the leech has, of course, a good deal to do with the length of time taken to digest a meal, a big leech taking longer than a small one. I should say that about two to six months was the time taken to complete the digestion of a meal. I have no idea how long elapses in nature between the completion of digestion and the searching for more food. All the leeches captured that I opened were empty of blood, but they could not by any means always be made to feed—I opened some of the ones that had refused, and found that they were empty. On the other hand a leech which had obviously fed on December 2nd fed again on February 1st: this beast was eaten by the tortoise, but it seemed to have been feeding and certainly left a wound. Curiously enough all the land leeches I examined were empty, and that was also Dr. Willey's experience in the jungle. This is possibly due to the fact that the fed leeches are not in search of prey, and therefore not so easily found.

On one occasion a curious example was given of how indifferent the water-leech is as to whether it feeds upon warm- or cold-blooded creatures. I had tried to make one of them feed upon a Saccobranchus infected with Trypanosomes with a view to discovering if any such change occurred as that seen in T. vittatæ, the leech steadily refused to feed. I then took it out of the tank, and it instantly attacked my hand making the customary little triradiate scar and drawing blood. It was made to desist, and then immediately put on to a tortoise where it quite contentedly made a large meal.

The experiments with the Diya kudella were now abandoned as what appears to be the true intermediate host was come upon.

Dr. Willey, while making some observations at Kesbewa, about eight miles from Colombo, found three Emyda with a number of small leeches attached to the back of the neck, the angles between body and limbs, and the region near the tail. He very kindly brought them to me at once. The leech is a very small creature. It attaches itself to the tortoise, but is most capricious about staying on it; I found it a most troublesome little animal to work with on that account. It is not very easy to keep it alive in captivity.

The leech belongs apparently to the genus Glossiphonia, and has a trick of lying together in little clumps; it is able to swim, and also covers the ground rapidly by walking under the water on its suckers in precisely the same way that the common land leech does on land.

This leech broods its young, but it usually seems to carry about only very few with it. I have frequently got them with one or two quite good-sized young ones, and remember on one occasion taking three parents, each carrying one young one, from a tortoise, and putting them into a watch-glass. They had all got detached and mixed up in the process, a short time later they were once more arranged in pairs, but I had no means of discovering if each parent had selected out its own offspring or had just adopted the first one it met.

The Glossiphonia is a very inconspicuous creature, is quite aquatic, and dies very soon if left out of water, which probably accounts for it not having been found sooner. The tortoises were generally brought in by natives, and it was always some hours before they were examined, and I expect in many cases the leeches had died and dropped off before they reached the museum.

From this time forward leeches of this species were got from time to time, but it was difficult owing to the relatively free habit of the beast and small size to get them in numbers. A good number were got for me by Dr. Willey from Hambentot in the south-eastern part of the Island. They came from milk tortoises (Emyda vittata) living in a bathing place; alongside of them were lake tortoises (Nicoria trijuga) with Ozobranchus[1] upon them.

The leeches seem to be specific to the two tortoises, only one Glossiphonia, an empty specimen, was found on the top of the carapace of a Nicoria; its presence was probably quite casual, just as I have occasionally found a stray Branchellion on a Tropidonotus (water-snake) which was living in a tank with Nicoria—they never fed on the snake.

The digestion in the Glossiphonia completes itself in about two to six days, according to the size of the leech, but I do not know exactly what period of time must elapse before the

[1] Ozobranchus, a species of this genus of leech is found in great numbers upon Nicoria trijuga. Mr. Harding states that this is a new species.

animal feeds again in nature. An apparently empty leech will sometimes quite refuse not only to feed, but to remain on the tortoise. Nevertheless, from observations on captive leeches, it does not appear to me to be more than a few days. The Glossiphonia show a marked tendency to get into the less-exposed corners of the body, such as the folds of skin at the back of the neck, round the limb bases, and under the tail. They were actually seen to enter the cloacal chamber which is a relatively large cavity in these tortoises.

The Glossiphonia, in contrast to the horse-leech, shows the Trypanosome very frequently in nature, in fact, the majority of the specimens are infected. And the parasite persists in empty leeches where no coloured matter is to be detected in the alimentary tract. I was never able to find the very earliest stages of the parasite in this leech owing to the difficulty of manipulation. It was neither easy to get the leech in a condition willing to feed nor to catch it at exactly the right moment after feeding. This was, of course, due to its small size and wandering habits correlated with the exceeding rapidity of the early changes in the Trypanosome.

In the most recently-fed animals at my disposal the parasite was already in the shape of a rather broad flagellate approaching the crithidial condition—the first two divisions had, in most cases, already occurred.

Thus a Glossiphonia which had fed on infected blood at some time between 8 a.m. on April 6th and 7 a.m. on April 7th was opened just after the latter hour. The Trypanosomes were already mostly in the shape of crithidia, but a very few were still in the rounded state just completing division. Some long slender forms, very narrow, with pointed posterior end, and the flagellum only reaching back to a little more than the middle of the body were already present. This indicates that the development is even more rapid than in the horse-leech.

These long forms were not, I think, left over from the previous meal as they were of a type not usually found at the end of digestion.

The course of the infection in the Glossiphonia appears to be in brief as follows:—The Trypanosome ingested with the blood develops in a few hours into a flagellate, rather rounded and broad in shape. It may grow very considerably in size, and adopt the Trypanosome condition, i. e. with the kinetonucleus posterior to the trophonucleus. Division still proceeds. Great variation in shape and size occurs in this middle period of digestion, and the relative position of the two nuclei varies very much even in the two products of one division. All stages from the round, rather dumpy crithidia to immensely long and very slender forms moving with great rapidity darting across the field in a flash are to be seen in the crop at the same time.

These different forms will be described in greater detail when the stained material comes to be considered. Division stages may also be seen, and these are often unequal.

Towards the end of digestion the type becomes much more uniform, and slender forms with little protoplasm and flagella hardly exceeding the length of the body seem to dominate to the exclusion almost entirely of other forms. These creatures very often have the kinetonucleus just anterior to and almost embedded in the trophonucleus. They seem at this stage, moreover, to have reached the limit of division as dividing figures were never found. It appears probable that death would now ultimately supervene unless injected into the blood of the vertebrate host.

Conjugation was carefully watched for, but no sign of it was found. It was expected to occur probably immediately after the first divisions in the leech or else possibly towards the close of the middle period of digestion.

The process of conjugation might of course occur at another point of the life-cycle, namely, at the time when the Trypanosome is injected from the alimentary tract of the leech into the circulation of the tortoise. If that were the case it would amply explain the elusive character of this process amongst Trypanosomes, as that is the one part in the cycle of a plasma dwelling form which it is almost impossible to investigate properly.

The question of the surviving of the Trypanosome in the leech has to be considered. It is a matter which is not easy to settle quite definitely, but I do not think that it occurs in this case. Leeches from uninfected hosts showed no parasites. I had a tortoise with only Hæmogregarines in its blood, and I never got Trypanosomes in leeches from this individual. As far as my observation goes (both as regards the investigation of the live creature and of sections) the parasite is never found outside the alimentary tract, and, while to be found in the intestine, is much more a crop parasite than, for instance, T. raiæ in Pontobdella.

How exactly the infection is transmitted to the tortoise in the act of sucking I do not know. But this much is pretty clear, the leech seems to suck by rhythmic contractions, that is to say, the suction is periodically inhibited.

I do not know if the Trypanosome, which is certainly slightly rheotropic, is sufficiently so to swim against the inward flow of blood during the suction time, but it might conceivably be able to do so in the intervals during which this is suspended. Further, the skin of the host must be pierced before suction begins, and it may be during this part of the process that the parasites are communicated to the vertebrate.

The habits of the Glossiphonia seem admirably adapted to the requirements of an intermediate host : its trick of wandering from one tortoise to another, its apparent conservatism in the choice of a host, and the relatively rapid digestion are all features favourable to the spreading of such a parasite as a Trypanosome.[1]

III. Observations on Stained Material.

So much then for the observations upon the live material. The material for staining was treated in various ways; films were made and dried in air, then fixed in absolute alcohol—or they were exposed to osmic vapour and then allowed to dry—or they were plunged while still wet into corrosive sublimate and acetic acid and treated by wet methods throughout. It is a matter of regret to me that I did not fix more material in this last way. The films were stained by various Romanowsky methods, or, in the case of wet films, in Heidenhain's iron hæmatoxylin or Ehrlich's hæmatoxylin.[1] The Trypanosome was also studied in section from the various organs.

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[1] See note on p. 693.

The drying method followed by the Romanowsky stain gives excellent results with certain types of object, but may at times give misleading pictures, more especially with more massive creatures. It is therefore advisable, where possible, to control the results with material treated by wet fixation; osmic films are very valuable, but are open to the same objection in the matter of drying. The drying method seems to flatten and spread out certain types of organism; on the other hand, the wet fixation of blood-films equally certainly causes the parasite to shrink. This is very marked in certain Hæmogregarine phases. Fixation is generally a choice of errors, but by the combination of the dry and the wet method a very fairly accurate idea of the nuclear structure of the organism may be obtained.

Trypanosoma vittatæ (Pl. 16, figs. 1—7) in the blood of the vertebrate host shows dense protoplasm, markedly alveolar with longitudinal striations, corresponding to the myonemata so clearly visible in the live state. These are not always equally conspicuous. There is no doubt that the adult Trypanosome in the blood of the vertebrate is possessed of quite a definite outer sheath or periplast—this is clearly visible in crushed specimens. The myonemata appear to form part of this structure.

The protoplasm has a tendency to stain deeply, whatever the method used; granules and protoplasmic inclusions are never, in my experience, visible at this stage in the stained preparations. In the live state there are sometimes bright globules to be seen in the protoplasm; they are not very commonly present, and this occurrence is quite casual. They may possibly be of a fatty nature, and be dissolved out by the alcohol with which the slide is treated. Whatever their nature, they have no visible equivalent in the stained preparations. The membrane presents the usual structureless appearance, and takes up the stains very faintly and evenly. In the Romanowsky films, and also, though less frequently, in wet hæmatoxylin material, a line is to be seen on the membrane, just immediately inside the flagellum; it suggests a supporting or skeletal structure and takes up the plasma stain.

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[1] I am indebted to Prof. Minchin for advice on the handling of these wet preparations.

The flagellum runs at the edge of the membrane, and can be traced back very close to the kinetonucleus, but not as a rule actually into it. A minute granule[1] can occasionally be seen just at the root, but it is not by any means always visible. The kinetonucleus is rod-shaped; in Romanowsky preparations it shows as a more massive structure than in the wet films treated with Heidenhain. It is always surrounded by a slightly clearer area of protoplasm, but there is no sign of a definite vacuole in its neighbourhood as is described for some of the mammalian Trypanosomes.

In a dried film the Trypanosome is practically presented in one plane, and a certain amount of widening out occurs, this causes a disturbance of the internal structures to a greater or less extent. The form in question during its sojourn in the blood of the vertebrate is, relatively speaking, a massive creature, it is oval in cross-section, as can be seen in paraffin sections from the lung. It is therefore not particularly well adapted for the drying method. In the wet films the trophonucleus shows a large chromatic karyosome surrounded by a clear area, which, in turn, is bounded by a sharply-defined ring, which takes on a nearly black colour with iron hæmatoxylin (Pl. 16, figs. 1 and 2). I propose to use the word nuclear membrane for this outer ring. I merely do this as a matter of convenience; it is not at all clear how far it can be regarded as a true nuclear membrane in the metazoan sense, and I do not wish the word to be taken as implying homology. I have never been able to see on hæmatoxylin preparations any strands passing from the karyosome to the membrane, although it is a very usual feature in this type of nucleus.

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[1] This corresponds apparently with the blepharoblast of Minchin. 'Q. J. M. Sci.,' vol. 52, 1908.

It is to be noted that the protoplasm shows no differentiation round the membrane of the nucleus, that is to say, that in the wet method films stained with hæmatoxylin there is no protoplasmic halo.

A slightly different appearance is presented in the dried films here (Pl. 16, figs. 3 and 4), there is a wide protoplasmic halo which always takes the blue stain of the Romanowsky combination much less deeply than the surrounding cell substance.

Approximately in the centre of this is the karyosome, which is composed of a blue staining substance underlying a reticulum of red staining chromatin. In some specimens strands seem to pass out from the karyosome—occasionally a pale (fig. 3) red ring surrounds the karyosome and strands may be seen passing between them. It is to be noted that this ring is well within the protoplasmic halo. My interpretation of the Romanowsky appearance is as follows :—The red ring corresponds to the membrane (in the Heidenhain preparations) which is generally destroyed in the drying.

What I have called the protoplasmic halo in the Romanowsky picture corresponds to the space between the karyosome and the membrane, but it is much widened by the pulling back of the protoplasm due to the general flattening of the whole organism.

As before noted small specimens are present, the origin of these (Pl. 16, figs. 5 and 6) is still obscure; they may either arise from the large forms by division or they may possibly be the young forms derived by infection from the leech. I am inclined to consider the former explanation as the more probable. It is, however, impossible, to form any very reasonable opinion on the point except from the results of experimental inoculation.

These small forms have hyaline protoplasm staining faintly with the blue of the Romanowsky combination. The protoplasmic halo is not visible in these forms, and in the wet preparation the membrane or outer ring is always rather faint.

The kinetonucleus lies very close to the anterior end of the body which tapers very rapidly to a sharp point. The membrane is relatively wide and the free flagellum is long.

Division stages in the adult Trypanosome must be exceedingly rare, as although a very large number of well-infected films have been searched I have never come across any of the full-grown forms in this condition. Among the forms intermediate between the adult and the small specimens, however, individuals with two nuclei and dividing kinetonuclei are to be found; they are never numerous, and I can say nothing of the details of the process (Pl. 16, fig. 7).

As already indicated I can find no morphological grounds for dividing the adult organism as found in the blood of the tortoise into male, female, and indifferent. There is no evidence to suggest that the small specimens like those shown in Pl. 16, figs. 5 and 6, are males and the large females, and outside of this the difference among the specimens is very slight, and involves apparently only protoplasmic features. This, in itself, is, however, no argument against an ultimate sexual differentiation or conjugation in the intermediate host.

The material from the leeches, I am sorry to say, was mostly fixed by the dry method followed by alcohol, a good deal being fixed by the osmic vapour method to act as a control. The Trypanosome is also to be recognised on sections of the whole leech, but it is difficult to get a very clear and brilliant picture. However, the dry method is better adapted to the thin leaf-like shape of the Trypanosome in the intermediate host.

The detail of the very earliest changes in the Trypanosome upon being taken into the leech is difficult to get in the stained preparations as they occur before the leech has ceased feeding. However, they are sufficiently clear from the live observations. As soon as the Trypanosome has rolled itself up and cast off its flagellum, the division of the nuclear elements begin, and it is at this stage that they are to be found in material from the crop. This early part of the work has mostly been made out from material from the Limnatis (figs. 8—12).

The two first divisions both of the trophonucleus and kinetonucleus follow quickly upon each other, the details of division being not so clear as in T. raiæ. Probably this is due to the slowness of the process in this last-mentioned Trypanosome, which in conjunction with the rich infections found in the intermediate host make the obtaining of a complete series a comparatively simple matter.

In the division of the trophonucleus of T. vittatæ there is a marked resemblance to what occurs in T. raiæ; in fact, the two processes are quite parallel. We have here also a nuclear division where no equatorial plate is formed, and where (figs. 9 and 10) no differentiation of the chromatin into chromosomes occurs. In both cases there is a well-marked spindle apparatus which appears to bring about the division of the chromatin with only a slight disturbance of its arrangement. So also in both instances the substance of the spindle apparatus is absorbed in the protoplasm and not enclosed in the reformed nuclei.

Fig. 9 shows a stage in this division process, and also fig. 10 in which the kinetonucleus has divided into four already, and both trophonuclei are in the act of undergoing the second division.

The kinetonucleus, however, shows a considerable difference from the conditions obtaining in T. raiæ.

In T. raiæ the kinetonucleus became greatly elongated, and then divided transversely, the products of division often remaining connected by a red staining band. In T. vittatæ the kinetonucleus when dividing splits through longitudinally. Sometimes the split starts at one end of the kinetonucleus, but takes a considerable time before being completed, and it may thus present a horseshoe shape. The kinetonucleus generally precedes the trophonucleus in division, but this is not by any means invariable. I cannot find any sign of the centrosomal function described by França for the Trypanosome in Hyla arborea.[1] He describes a development of the Trypanosome resembling this early process in the leech, but in the case studied by him the kinetonucleus appears to enter into what he considers definite centrosomal relations with the trophonucleus at the time of division. This is quite absent in T. vittatæ.

As already stated the flagellum develops with no very close regard to the exact stage of the nuclear divisions. It grows out rapidly, and in the stained preparations seems to develop by direct outgrowth from the kinetonucleus. The complicated figures met with at a certain stage in the development of T. raiæ in Pontobdella are here entirely absent. The products of the divisions show a certain amount of variation as regards the state of their development at the time of being set free. Pl. 16, fig. 11, is a very typical example of the more rounded forms, fig. 12 is another, and a third is shown in fig. 12 *a*.

Twenty-four hours after feeding the parasites are all flagellate forms varying considerably in shape, but most of them already showing an undulating membrane, and the kinetonucleus has migrated as a general rule pretty far back though it is still in front of the trophonucleus (Pl. 16, figs. 11—13, 16, and 17). In the Glossiphonia this process is still quicker, and from this time on the infection in this leech begins to show the very wide range of forms so characteristic both of T. vittatæ and T. raiæ.

A review of Pls. 16 and 17, figs. 13—26, will give some idea of the various types present. Long slender forms, some with exceedingly elongated bodies and the kinetonuclei showing considerable variation in their relation to each other, typical Trypanosomes often somewhat broad in shape, and small dumpy forms with short flagella, are all present together at

[1] ("Recherches sur les Trypanosomes des Amphibians." 'Arch. de L'Inst. Roy. de Bact. Camera Pestana.' T. i, Fasc. ii. 1907).

this middle stage of digestion. From such a range of forms it would be a simple matter to pick out creatures suggesting the morphological features which are considered characteristic of male and female individuals. Thus Pl. 17, figs. 23 and 25, might be regarded as respectively male and female; so also Pl. 17, figs. 24 and 26; but until the act of conjugation is observed I cannot see that there is evidence enough to make it clear that the quite obvious morphological difference is the expression of a sexual differentiation.

At this middle stage of digestion dividing individuals are of frequent occurrence. In Trypanosoma vittatæ unequal division at this stage is the rule rather than the exception. The process is figured in Pl. 17, figs. 18 and 19, and the different character of the products is obvious.

The new flagellum unquestionably grows out apart, and does not arise by splitting off from the old one. The splitting of the flagellum, as is well known, does occur in certain Trypanosomes; as, for instance, in T. grayi described by Prof. Minchin,[1] and it is of interest to find two such very different methods holding good in the group.

The new flagellum in T. vittatæ will start growing out from the kinetonucleus before it is obviously divided, though it is generally enlarged. A review of Pls. 16 and 17, figs. 16—20, will demonstrate this question pretty clearly.

As time goes on the nature of the infection undergoes a very marked change; the predominating type of Trypanosome is now found to be a slender rather short form with the flagellum extending only to a short distance beyond the end of the body. The undulating membrane is very narrow. The tropho- and kinetonuclei have entered into close relations with each other, and generally the kinetonucleus is just anterior though closely applied to the trophonucleus. Specimens are found with the kinetonucleus posterior, but these are few in number. The gradual predominance of this type to the exclusion of practically every other can be

[1] "Trypanosomes in Tsetse flies and other Diptera," 'Q. J. Micr. Sci.,' vol. 52, 1908.

followed very clearly in different infections. Thus Pl. 17, figs. 31—33, came from a quite empty leech, and only this type with very slight variations was present. Pl. 17, figs. 18—27, come from a leech in the middle period of digestion, and this final type is just beginning to appear in isolated specimens. In another leech a little further advanced it is more numerous than any other, but not exclusively in possession of the field.

I have never seen division in this type which appears at the end of digestion. I am inclined to think, although there is no actual evidence, that these forms die off unless injected into the blood of the vertebrate.[1]

Exactly what happens to the forms which disappear during the course of digestion is not very clear. The broad forms, however, appear to give rise to such forms as Pl. 17, figs. 29—33, simply by division and direct development. The case of the very long slender forms (Pl. 17, figs. 25, 26, and 28) is more difficult, and the material at my disposal does not throw much light on the question.

IV. GENERAL REMARKS AND CONCLUSIONS.

The life-history of Trypanosoma vittatæ shows a close resemblance to that of T. raiæ. Some quite recent observations upon this last mentioned Trypanosome made in November, 1908, at the Millport Marine Station with Pontobdella which I had reared from the egg, have amply confirmed Brumpt's brief sketch of the early stages of the life cycle.[2]

Moreover, skate's blood, which contained the Trypanosome kept upon sealed slides has shown that here also the Trypanosome discards its flagellum and undergoes a number of divisions. The process is similar to that of T. vittatæ, but the rounded non-motile stage seems very much more persistent. In the Pontobdella motile flagellate stages do not begin to appear for four to six days, although the flagellum itself may be present for some time, apparently more than a day, before it becomes motile. The earliest divisions of the rounded Trypansome, instead of following each other immediately as in T. vittatæ, are here at intervals of at least twenty-four hours. The early stages of development of the flagellum are also very slow.

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[1] It is very difficult to be certain that division never takes place in this form, but I have never seen any sign of it.

[2] 'C. R. Soc. Biol.,' ix, 1906.

This process by which the Trypanosome rounds itself off and after a number of divisions produces a definite Crithidial flagellate, appears to me to be more widespread among Trypanosomes of a certain type, notably those from cold-blooded hosts, than it has hitherto been considered.

França observed an analogous process some time ago in the Trypanosome of Hyla arborea, also in T. granulosum.[1] Brumpt describes it for this Trypanosome also in the leech Hemiclepsis, and Dutton, Todd, and Tobey[2] have seen it in Trypanosoma loricatum.

Another point of interest in the life cycle of T. vittatæ is the very marked development of a uniform slender type of parasite at the end of digestion. In T. raiæ this also occurs, though here some rounded forms seem often to persist. A somewhat similar development occurs in T. grayi,[3] where the last stage of the infection shows, however, two slender forms, one of which, a Herpetomonas-form, encysts in the proctodæum of the Glossina, while the other does not encyst, and seems probably to be destined to transmit the infection by inoculation into a vertebrate host.

I need not emphasise the absence of evidence of conjugation. This process if it occurs seems particularly difficult to observe in Trypanosomes, and no quite satisfactory account of it has yet been given for this group of flagellates.

It is a point of only slight interest, but it is curious to note to what an extent the infection is capable of persisting in the Limnatis, which is apparently not a true transmitting host.

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[1] 'Bull. Soc. Portug. Sc. Nat.,' vol. i, p. 3, Dec., 1907.

[2] 'Ann. Trop. Med. and Parasit.,' vol. i, No. 3, 1907. (I have not seen the original memoir.)

[3] Minchin, loc. cit.

The work here recorded was done partly at the Government Museum in Colombo, Ceylon, and partly in the Zoological Laboratory in the University of Glasgow. I am much indebted to Prof. J. Graham Kerr for kind suggestions during the course of the work.

Glasgow; December, 1908.

Note.—It may be objected that no absolute proof has been adduced that the form in the Glossiphonia is T. vittatæ. Absolute proof was not possible from the nature of the conditions, but the evidence seems to me to be very strongly in favour of the identity of the parasites. The correspondence of the early stages in Glossiphonia with those in Limnatis where experimental feeding could be carried out, the final stage in the Glossiphonia at the end of digestion being a slender flagellate and not a rounded-off organism as occurs in the flagellate of non-bloodsucking insects, and the absence of the parasite in the leeches from the tortoise where no Trypanosomes could be found all seem to me to point to the stages in Glossiphonia being true developmental stages in the life cycle of T. vittatæ.

I may add that no flagellates were ever found in the land leech or in Ozobranchus or in Limnatis. Ozobranchus, of which a very large number were examined, lives in exactly the same habitat as Glossiphonia but is parasitic on Nicoria trijuga instead of Euyda vittata. Nicoria never showed a Trypanosome.

EXPLANATION OF PLATES 16 AND 17.

All figures were drawn with the Abbé camera under a 2 mm. Zeiss apochromatic immersion objective. A No. 12 compensating ocular and tube length of 250 mm., giving a magnification of approximately 3600 diameters. This has been reduced by the lithographer to approximately 2400 diameters.

Figs. 1–7.—Trypanosoma vittatæ from the blood of Emyda vittata.

Fig. 1.—Trypanosome from blood of tortoise stained with Heidenhain's iron hæmatoxylin after fixation with corrosive and acetic, wet method throughout.

Fig. 2.—As above, showing a characteristic attitude.

Fig. 3.—Dried Giemsa film of Trypanosome showing protoplasmic halo and red ring round karyosome.

Fig. 4.—Trypanosome as above, showing myonemata and line along undulating membrane.

Figs. 5 and 6.—Small specimens from blood of tortoise.

Fig. 7.—Dividing stage from blood of tortoise.

Figs. 8–12A.—Early stages in crop of leech. These are from the water leech, Limnatis granulosa.

Fig. 8.—Division stage; the four new flagella are already developed; kinetonuclei are dividing by longitudinal splitting.

Fig. 9.—Division stage showing nuclear spindle.

Fig. 10.—Second division occurring before the completion of the first as regards the protoplasm. Both kinetonuclei in act of division, only two flagella so far developed.

Fig. 11.—Rounded flagellate—the product of the divisions of the rounded off Trypanosome.

Fig. 12.—Early flagellate stage; note the lengthening of the body.

Fig. 12A.—Another early flagellate stage.

All the remaining figures are from the Glossiphonia, with the exception of 16 and 17.

Figs. 13–15.—Osmic fixed film from leech just about the beginning of the middle stage of digestion.

Fig. 13.—Flagellate stage showing elongated body.

Fig. 14.—Flagellate, with broad posterior end.

Fig. 15.—Very long flagellate kinetonucleus at same level as trophonucleus.

Figs. 16, 17.—Early flagellate stages from horse leech to show secondary increase in size and preparation for division. Note the condition of the flagella showing outgrowth from the kinetonucleus.

Figs. 18–27 from the Glossiphonia at middle stage of digestion.

Fig. 18.—Division stage. Note relative position of the kinetonuclei and the condition of the flagella. The unequal character of the division is obvious.

Fig. 19.—Another division stage. The features are much as in Fig. 18.

Fig. 20.—Early division stage to show condition of kinetonucleus and flagella.

Fig. 21.—Trypaniform individual with broad posterior end and many red-staining granules in the protoplasm.

Fig. 22.—Small broad form.

Figs. 23 and 24.—Short, rather broad, trypaniform individuals.

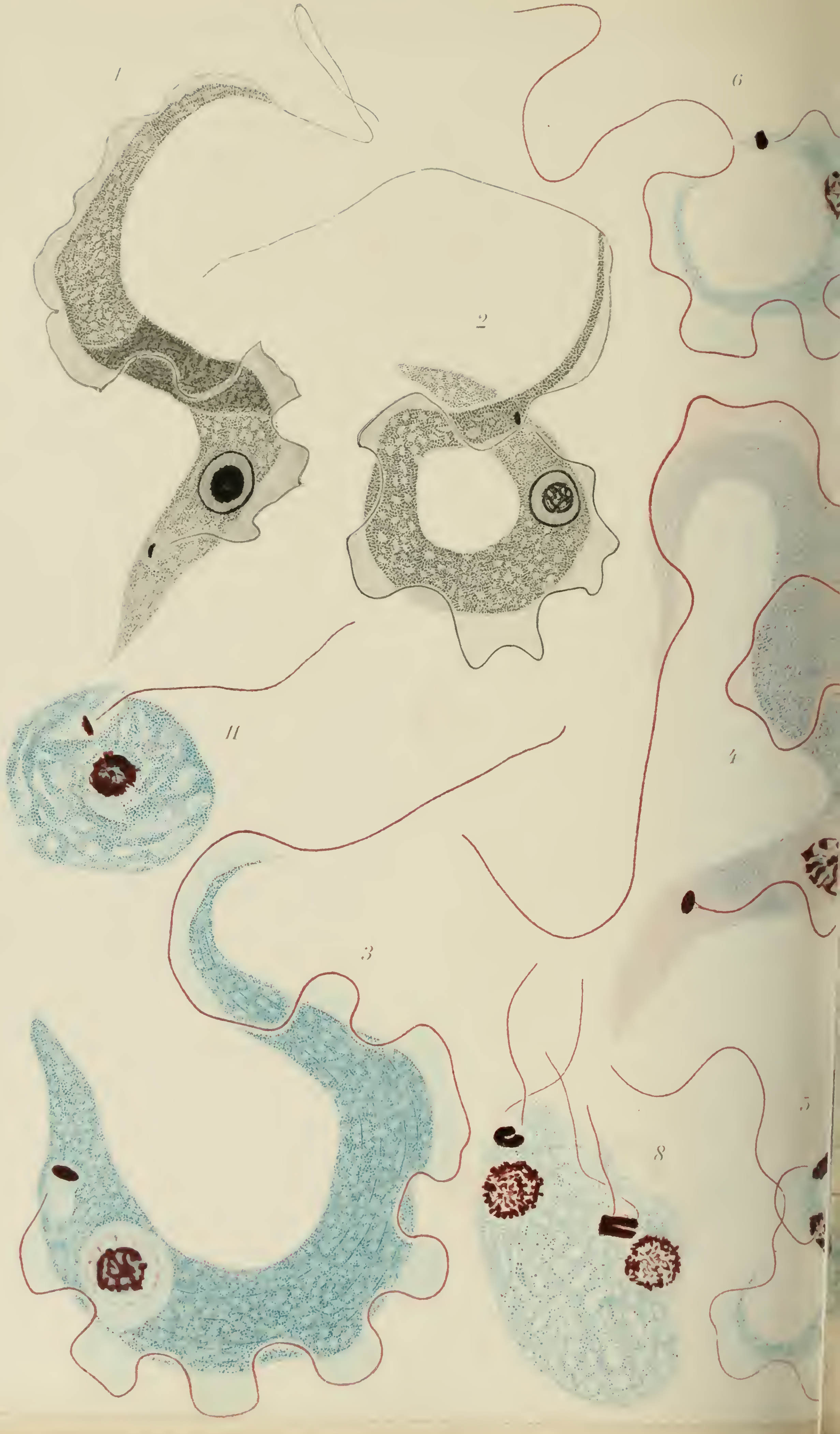
Figs. 25 and 26.—Long slender forms.

Fig. 27.—Isolated forms of this type are just appearing in this leech which is at the middle stage of digestion. This is very like the final type developed at the close of digestion.

Fig. 28.—Very long slender form.

Figs. 29 and 30.—From a leech whose digestion is still more advanced Note the difference in the type of the organism.

Figs. 31–36.—Flagellates from leech at end of digestion. this type alone is present with very few exceptions.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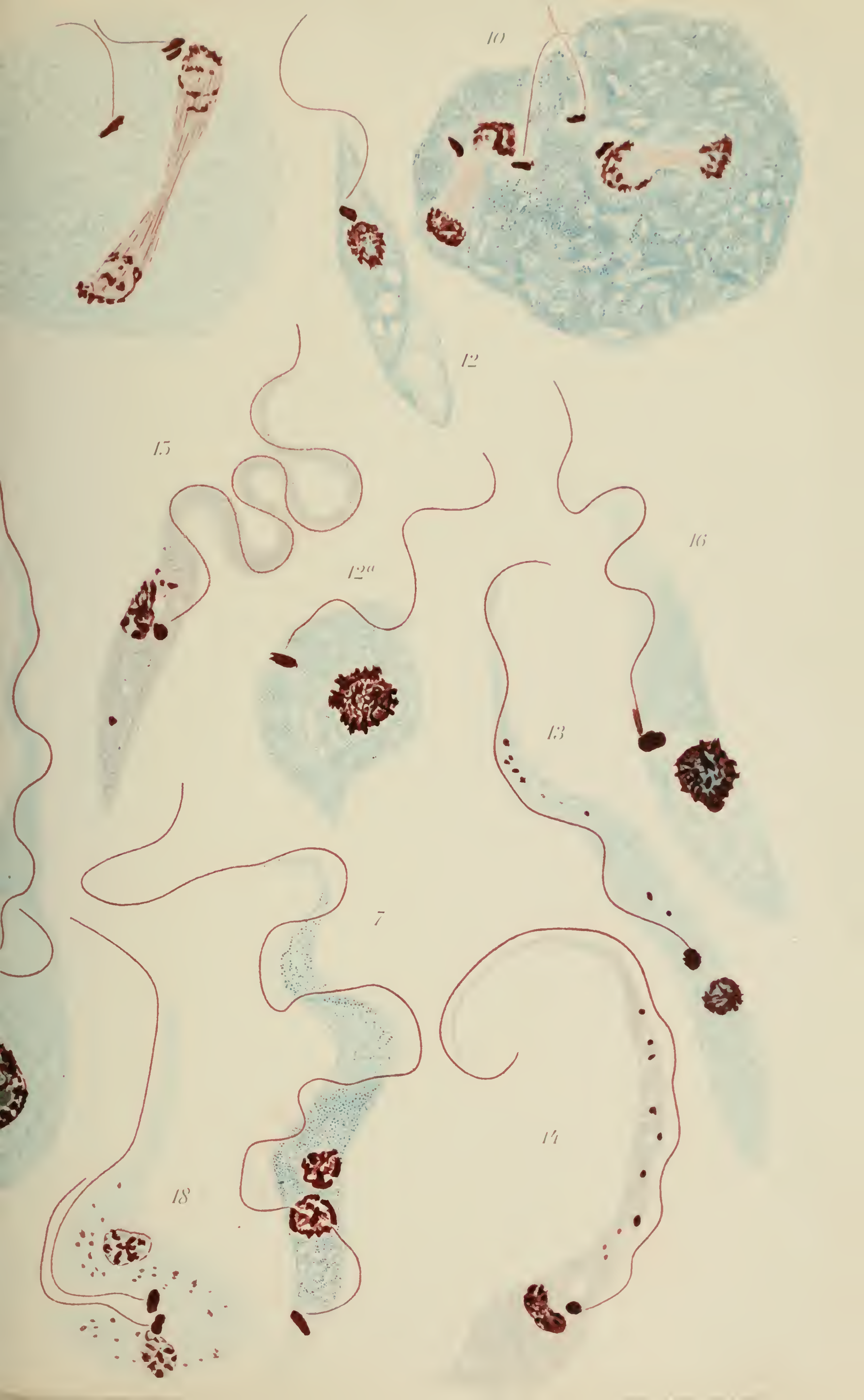
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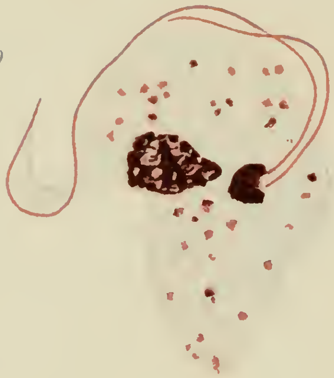

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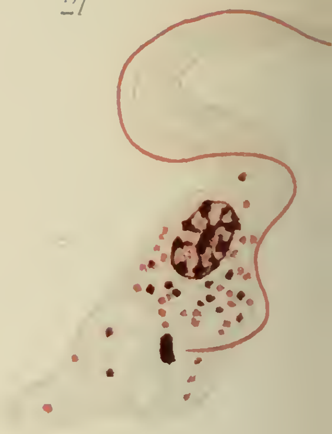

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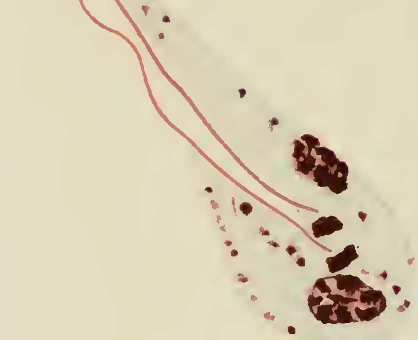

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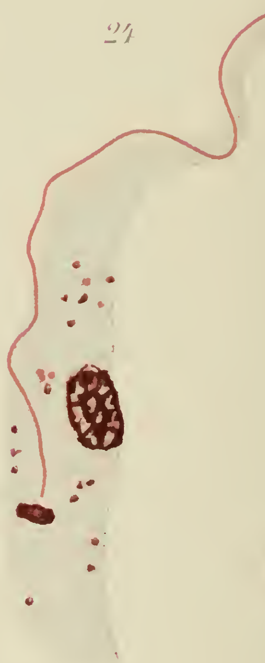

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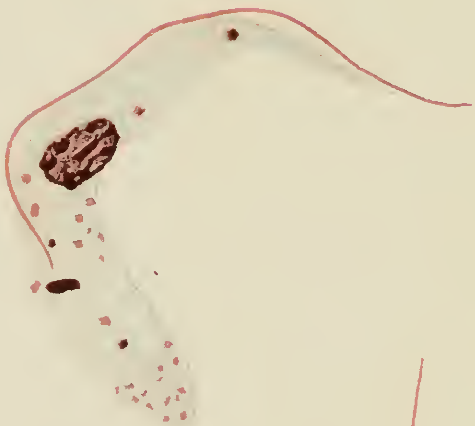

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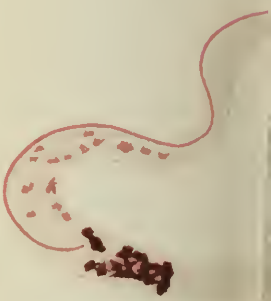

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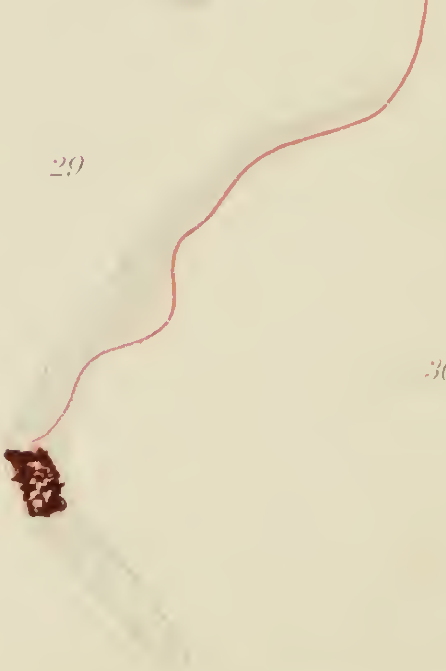

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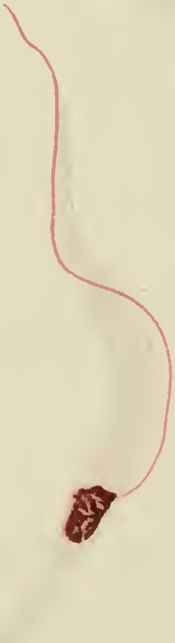

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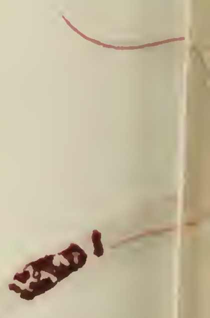

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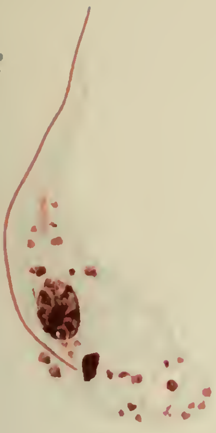

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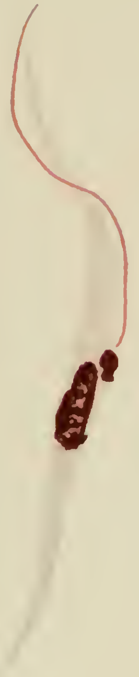

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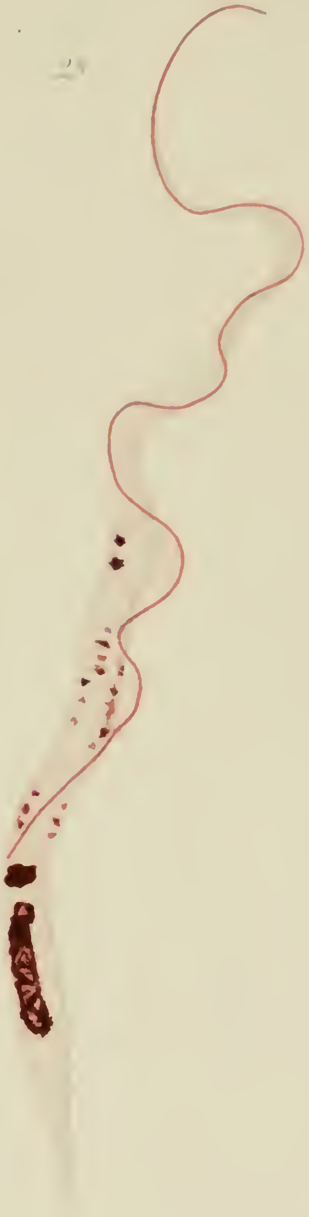

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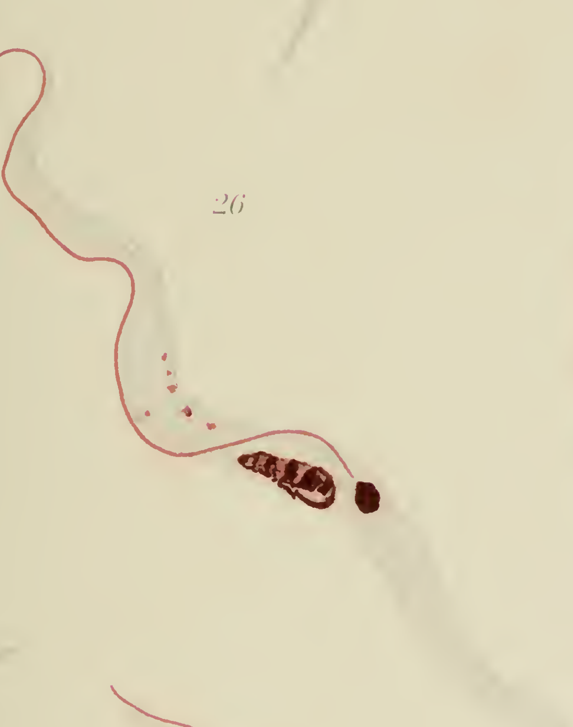

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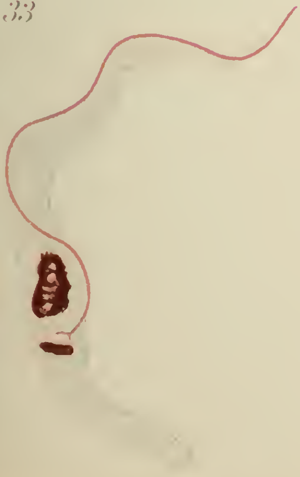

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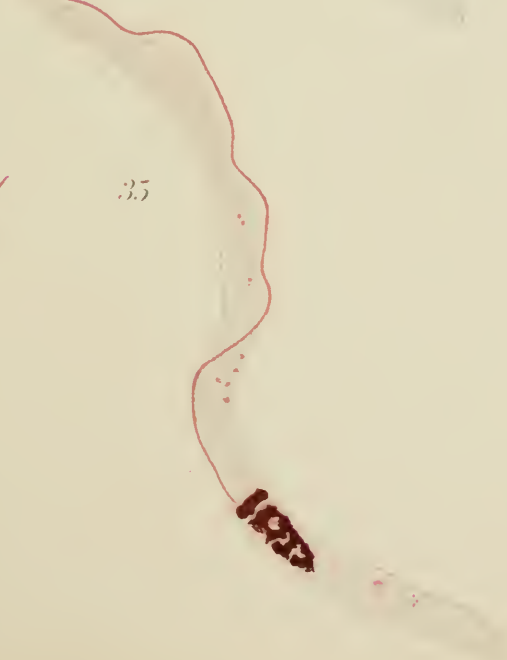

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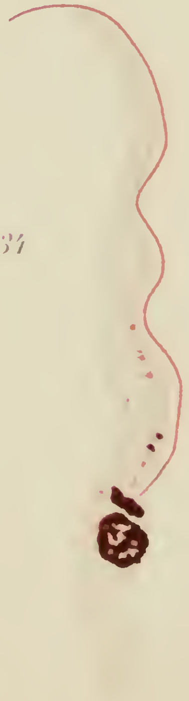

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Further Notes on a Trypanosome found in the Alimentary Tract of Pontobdella muricata.

By

Muriel Robertson, M.A.,
Carnegie Research Fellow; Junior Assistant to the Professor of Protozoology in the University of London.

With Plate 9 and 5 Text-figures.

INTRODUCTION.

In a previous paper published in the 'Proceedings of the Royal Phys. Soc. of Edinburgh,' vol. xvii, No. 3, 1907, an account was given of the main features in the life cycle of T. raiæ as it appeared in the leech Pontobdella muricata.

I could not, however, at that time get clear knowledge of the early stages of the infection in the leech, nor could I get conclusive evidence that the form appearing in Pontobdella was really Trypanosoma raiæ. The following observations fill in these important gaps, and appear to me to make it clear that the flagellates found in such abundance in this leech are derived from the skate and form part of the life cycle of T. raiæ.

I have also taken the opportunity afforded me by a fresh set of infected leeches of again working through many of the stages in that host. On this occasion I have abandoned the Giemsa method and the dry fixation of the films, and have used instead such fixatives as Schaudinn's fluid, Hermann and Flemming's fluid. The stains used were iron hæmatoxylin, Delafield's hæmatoxylin, Twort's combination of licht-grün and neutral red, and acid fuchsin. These are all useful stains. The Giemsa dry film method has been so much criticised that one felt that cytological work carried out with this stain was on an insecure basis until confirmed or criticised by other methods.

The corrections in this paper apply only to cytological detail, and my view of the life cycle, as a whole, has only received further amplification from the new facts at my disposal.

In the spring of 1907 I received a number of Pontobdella cocoons from the Marine Station at Plymouth; they had been deposited on clam shells. I put them into a glass jar filled with clean sea water, covered it with a loosely-fitting glass lid, and simply put it on to a shelf in the laboratory. I went to Ceylon in the course of the summer, and was later informed that the leeches hatched out about the beginning of October, 1907, although the exact date was not noted. On my return in the autumn of 1908 they were still alive in the original jar and the same water. In November I took them to the Marine Station at Millport,[1] on the Clyde, and fed them on infected skate to obtain the first stages of the parasite in the leech.

I may say in passing that the Trypanosome infection is very common in the skate caught in the Clyde area; more than 50 per cent. of these fishes are infected, a circumstance which accounts for the great frequency of the parasite in the leech, only occasional specimens being uninfected.

The leeches, it was observed, showed the greatest excitement —waving about actively in all directions—when a shadow was made to fall upon the jar containing them. This reaction to the appearance of a shadow is very marked indeed, and must be of service to the creature in its natural state. The skate, coming over the ground upon which a leech is attached, will, of course, cast a shadow, and this will be one of the ways the leech becomes aware of the presence of its host.

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[1] I wish to express my thanks to the director of the station for affording me all possible facilities in carrying out these experiments.

The young leeches attached themselves readily enough to the skate, except in one or two cases where they required a good deal of coaxing; ultimately, however, they all fed.

The Pontobdella apparently does not feed at once, possibly on account of the difficulty in piercing the skin. The attitude of the leech when attaching itself with the intention of feeding is very characteristic. It takes firm hold with its posterior sucker, and then bends its anterior sucker in so that it is touching its own body. It then slides the anterior sucker down along the body, finally attaching it to the skate at a spot close beside the posterior sucker. These leeches made quite a large wound for such small creatures.

In some cases, after feeding for a time, the leech detaches the anterior sucker, but does not, as a rule, remove the posterior one. Finally, after a greater or less interval of time has elapsed, the feeding is resumed. On one occasion I was able to observe the process very clearly. A leech was put on to a skate at 2.30 p.m., on the 14th November, 1908; at 10 a.m. on November 15th it was attached by the posterior sucker only. The leech had fed, but had not taken very much (the leech is, of course, rather transparent when young, and it is possible to tell at a glance if it has fed or not). Three quarters of an hour later it was still in the same position, with the anterior sucker free. I then held the skate under water in the tank in such an attitude that I could see the leech, which had begun to make searching movements with the anterior sucker. It presently slid the anterior sucker down its body and on to the skate, but moved it about until it passed over the old wound, when it buried its sucker in it. The leech seems to force its proboscis in pretty deep, judging from the wound and its attitude while sucking. At 7 p.m. on the 15th the leech was again only attached by its posterior sucker, but it was much swollen, and had blood right up to the anterior end. It was left attached to the skate till the morning of the 16th, but did not seem to feed again.

Anyone who has reviewed a large number of a given species of leech will have observed that there is a good deal of individual variation in the processes of digestion. The broad lines are of course the same, but there is always a certain amount of individual idiosyncrasy. This in Pontobdella is chiefly expressed in the greater or less fluidity of the blood in the crop and the nature of the bacterial flora moulds, schizomycetes, etc., present. These complex circumstances no doubt react upon the Trypanosomes and may explain a certain variability in the detail and also in the time co-efficient of some of the developments.

The blood in the crop of Pontobdella has a tendency to coagulate. It forms a rather dry mass with fluid in the interstices. The time factor in this stiffening of the blood is rather variable. It always occurs, but the time at which it happens and the length to which it goes differs a good deal in individual specimens. Late in digestion the mass in the crop tends to become fluid again.

In Pontobdella the crop is a single rather thin-walled sac passing back from the œsophagus right to the posterior end of the body. The opening from the crop into the intestine is placed at a point about two thirds of the way from the anterior end and it passes back as a narrow tube lying on top of the crop.

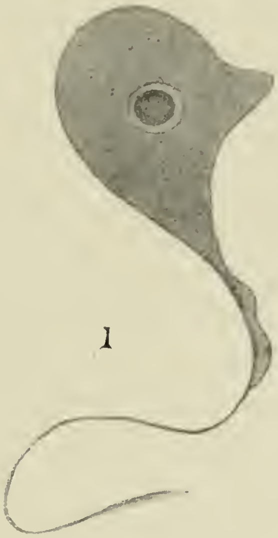
The young Pontobdella which had been hatched in captivity were opened at different intervals after feeding on infected skate.

The condition of the parasites was carefully observed and the following course of development was found to take place. It must be observed in passing that skate's blood contains an immense number of leucocytes, which present very confusing appearances; also the parasites are rather scarce until the multiplication period sets in. Some searching is therefore required to find the Trypanosomes in the earliest stages.

A leech (*5a*) was put on to an infected skate at 9.30 p.m. one evening; 12 hours later it was feeding, and had already ingested a good deal of blood; 2½ hours later it had ceased feeding. The leech was opened 17½ hours after it had been first put on to the skate, and 3½ after it had finished feeding. So that the earliest ingested Trypanosomes had been in the leech about 16—17 hours, and the last ingested ones about 3—4 hours.

The blood was very fresh-looking, and no obvious changes had taken place in the blood corpuscles. The Trypanosomes showed very variable appearances. A good number still showed the flagellum, but were no longer in the typical Trypaniform condition. For the most part they were somewhat pyriform (text-fig. 1), with an immensely long, thick flagellum protruding from one end. Some very fantastic appearances were seen where the body of the Trypanosome had assumed an irregular shape with curious rounded bulges, and where the flagellum had broken loose from the membrane, and had become tangled round the body, the end was usually free

Text-fig. 1.



Drawing of live Trypanosome from the crop of Pontobdella. The animal, which has been recently ingested with the blood, is in process of rounding off.

and still motile. In other cases free flagella still actively motile were seen; this has often enough been observed with Trypanosomes, but in this case the flagellum does not take the kinetonucleus with it. Uninucleate stages of this parasite have never been seen. One of these free motile flagella was seen to become secondarily attached to a resting individual. This animal was watched for many hours in case the process might prove to be of more than merely casual significance, but no development took place. Besides these flagellate creatures, others were present which had already discarded the flagellum (text-fig. 4). These were rounded egg- or pear-shaped individuals with a characteristic clearly visible nucleus. It is composed of a softly refractile circular body surrounded by a bright halo. The nucleus lies towards the broader end of the body. These animals present a very characteristic appearance, but, nevertheless, are easily overlooked in the mass of leucocytes and blood-corpuscles.

These non-flagellate organisms were already in a few instances undergoing division but no sign of the new flagellum was as yet forthcoming.

Another leech (7*a*) opened forty-eight hours after it began to feed showed only resting forms. The blood in the crop had coagulated into a rather dry mass but the corpuscles showed no signs of degeneration.

These resting-stages of the Trypanosome are identical in appearance with those so frequently seen in Pontobdella found infected in nature. A resting pear-shaped individual was chosen as a subject for observation at 4.30 p.m. When it was first observed[1] the trophonucleus was clearly visible and had its usual appearance of a sphere surrounded by a halo. Half an hour later the animal was more rounded, the nucleus was less distinct, and a slight groove had appeared at the broad end. By 5.30 the nucleus, as such, had disappeared, but a large clear oval space had appeared in its stead. At about 5.50 the two nuclei began to reappear, joined by a clear area. At 6.15 the two nuclei were once more quite clearly defined but the clear area joining them remained visible till about 9 o'clock after which it was no longer to be detected. This clear band is the remains of the division spindle: it is a very characteristic feature in the stained specimens. During the division of the nucleus the body had gradually become flattened in an antero-posterior direction and correspondingly widened laterally. Grooves also began to appear in the antero-posterior direction. It is to be noted that these arose both at the anterior and the posterior end—the anterior end being the broad end at which the nucleus lies when the animal is in the pear-shaped condition and at which the flagellum is later developed.

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[1] I am indebted to Mr. C. H. Martin for kind assistance in carrying out some of these continuous observations upon the live specimens.

These grooves altered a good deal in appearance during the next few hours and towards 4 a.m. had deepened till the animal presented the picture of two pears stuck together in the middle, with, however, the two broad ends and the two pointed ends free. This is a point of some slight importance. When in the trypaniform state division of the protoplasm of this parasite usually begins from the anterior end and proceeds to the posterior. This is likewise the rule in the crithidial state. This question of the grooves arising at both the ends of the parasite is not in itself deserving of much remark but it explains some curious stages where division of the protoplasm goes from the posterior to the anterior end, to be described in a later part of the paper.

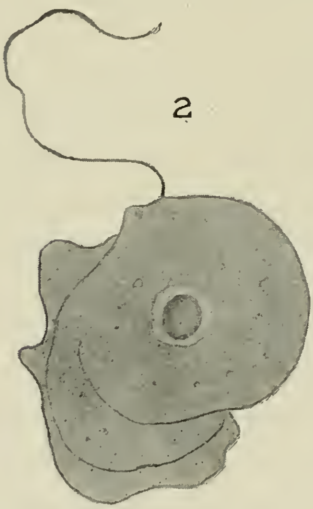
This individual was watched for another two hours and one of the two daughter-individuals developed a stiff and very short flagellum which, however, showed no signs of movement. The other individual appeared to have a little projection suggesting a flagellar rudiment. At 6.30 a.m. the animal was finally abandoned, although the complete separation of the protoplasm had not yet occurred.

The flagellum seems to appear for the first time somewhere between the second and third day after feeding. It is a very characteristic feature that it generally arises at a division stage. The flagellum is at first a stiff and relatively thick little rod which sticks straight out from the anterior end of the organism. A very considerable time seems to elapse before it becomes motile, I cannot say exactly how long, but it seems to be more than twelve hours.

A leech (6*a*) opened six days after feeding showed a typical infection of the varied type so characteristic of Pontobdella.

True Trypanosomes had already appeared, some of these were broad individuals and others were much more slender, but not of the elongated type which appears at a much later stage of digestion. The broad and slender types were joined up by innumerable intermediate forms. Besides these, crithidial forms were also present. I call crithidial forms those where an undulating membrane is developed, but which have not the typical Trypanosome arrangement of the kineto- and tropho-nucleus. Herpetomonad forms with the flagellum sticking straight out from the broad end of the body and with as yet no membrane were also to be seen. And finally

TEXT-FIG. 2.



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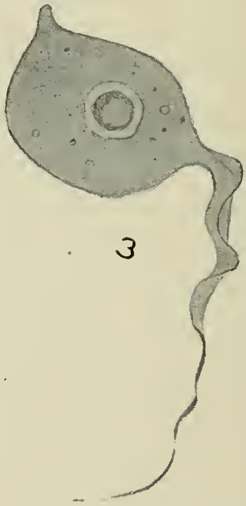
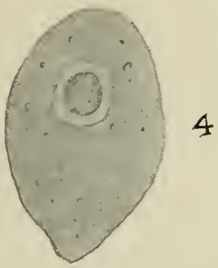


Trypanosomes from the blood of the skate rounding off on a sealed slide. The drawings are from live specimens.

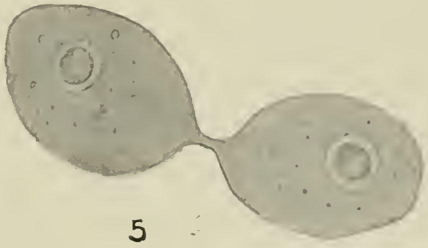
many rounded forms, some dividing and some developing the flagellum, were likewise present. Some of the trypaniform individuals were already dividing.

Conjugation was very carefully searched for as it seemed probable that it might occur at this stage of the life cycle, but no signs of such a process were detected. Two individuals were found joined by their posterior ends, one slightly broader than the other. They were watched continuously from 6 p.m. till 3.15 a.m., and the protoplasmic junction between the two was seen to become much more slender and pulled out, showing that the individuals were dividing.

Text-fig. 4.



Text-fig. 5.



Text-fig. 4.—Rounded off Trypanosome.
Text-fig. 5.—Division of resting Trypanosome.
Drawings made from live specimens in a sealed slide of skate's blood.

An interesting corroboration of the stages above described was obtained from blood drawn from a skate and sealed up between a coverslip and slide (text-figs. 2—5). A Trypanosome was continuously watched from 2.45 p.m. when the slide was made. At 4.30 the animal had come to rest. The flagellum which, when it breaks free from the membrane, is seen to be of a relatively immense length was tangled up round the animal. The slide was watched for some hours longer, but as the Trypanosome was no longer motile it was left. Next morning it was found to have divided into two.

The behaviour of the Trypanosomes on a sealed slide is interesting—a number do not alter at all, others very soon after the slide is made begin to react to the altered conditions. They adopt a dumpy spiral shape, or the posterior end may become much thickened at the expense of the rest of the body (text-figs. 2 and 3). Sometimes the most fantastic shapes are seen, finally the flagellum breaks free but may remain attached to the Trypanosome by its posterior end. It may then become tangled round the body and stick out in stiff loops. The Trypanosomes in these phases on the sealed slide made from the skate's blood are absolutely identical in appearance with the motile forms described in leech 5*a* (cf. text-fig. 1). The time at which the Trypanosomes come to rest varies much.

On another occasion a rounded non-motile Trypanosome which had discarded its flagellum was chosen for continuous observation on a slide of skate's blood which had been mounted for fourteen hours. The animal was seen to divide into two about 11 o'clock in the forenoon. During the afternoon, at about 4 o'clock, these two individuals each divided thus forming four little rounded animals lying more or less in contact. By 7 the same evening they had become more oval and were identical in appearance with the resting phases in the leech. By 9.30 p.m. short projections were seen at the broad end of two out of the four creatures under observation. The slide was left about 10 o'clock that night as the animals were not motile. Next morning at 9.30—thirty-six hours after mounting the slide, observation was again resumed and it was found that the creatures had each divided. The eight resulting individuals were still closely apposed but not connected with each other. Unfortunately these creatures were lost owing to a careless movement. The slide was, however, in the following condition. Unaltered Trypanosomes, still actively moving, were to be seen, non-motile groups of four and also a few groups of six and eight individuals were present.

I had often noticed that the Trypanosomes on a sealed slide of skate's blood altered their shape, but, thinking this was merely a pathological manifestation, had not persevered with the observation. The process is easily enough passed over unless the observation is continuous, as the infections are generally rather slight, and, once the Trypanosome has come to rest, it is not quite a simple matter to see it among the large number of leucocytes and red corpuscles. Moreover, the curious fact that all the Trypanosomes on a slide do not round off leads one to imagine that no development has taken place.

A low temperature seems to favour the process. This work on the live skate's blood was carried out at the Millport Marine Station, and the most successful set of observations was obtained in very cold weather, when the temperature of the laboratory was much lower than usual.

It appears to me that these experiments with the young leeches, which confirm Brumpt's short sketch of the life cycle of T. raiæ, are good evidence that the Trypanosome in Pontobdella is T. raiæ. Of course it may be urged by those who do not consider that Trypanosomes have a cycle of evolution, however simple, outside the vertebrate host, that the young leeches were hereditarily infected with a flagellate of their own. Against this I can only advance that the long fast of more than a year, to which these newly hatched leeches were subjected, is not in favour of the survival of such parasites; and that the close correspondence between the early stages in the leech, and those obtained upon the sealed slide from the skate, is strongly in favour of the identity of the parasites. Further, even if, in addition to the direct infection from the skate, a hereditary infection also exists in Pontobdella, it has still to be proved that the form inherited is not T. raiæ.

The only way in which quite conclusive proof of the question could be obtained is by means of experiments with both leeches and skate hatched in captivity. I hope ultimately to be able to carry these out, but the difficulties in the way are obvious, as the obtaining of Pontobdella cocoons is a matter of chance, and the breeding of skate takes time and is often uncertain.

I cannot agree with Capt. Patton and Mr. C. Strickland, who, on the basis of my previous description, consider this parasite to be an independent Crithidia having no connection with T. raiæ. In a recent paper[1] I observe that they place it in this group with the name of C. robertsoni. I would like to point out that, quite apart from the question as to whether this parasite belongs to the cycle of T. raiæ or not, it certainly is a Trypanosome. The Crithidial, like the Herpetomonad stage which precedes it, is transitory; the animal ultimately adopting the Trypanosome state. I admit at once that the distinction between a Crithidia and a Trypanosome is not a very important one, but, such as it is, I do not see that there is any scientific point in neglecting it.

I now wish to give a brief account of the parasite as seen in stained films, fixed, for the most part, in Schaudinn's (alcohol-acetic corrosive sublimate) fluid. The stains used were Delafield's hæmatoxylin, Heidenhain's iron hæmatoxylin, Twort's licht-grün, and neutral red combination and fuchsin.

Delafield gives an excellent result, staining the nuclear structures with clearness and precision; the flagellar apparatus stains, but does so a little faintly.

Iron hæmatoxylin gives a very clear picture, staining the nuclear parts coal black, and bringing into good relief flagellar and cytoplasmic detail. Great care, however, must be taken in the interpretation of this stain, as it leads one into much the same errors as Giemsa's method, in so far as it stains chromatic and achromatic structures alike. Therefore, while Heidenhain's method gives a really splendid picture, it is necessary to check the results by Delafield's hæmatoxylin, which is a much safer stain.

Twort's combination of neutral red and licht-grün was also used. This is a clear, transparent stain, giving a red reaction for chromatin, and a green reaction for cytoplasmic and achromatic structures. The drawbacks to this stain are the uncertainty of action which seems to attach to all delicate double stains, and the fact that there is another loophole for uncertainty in the process of washing out the stain; so that it remains doubtful in some cases whether the nuclear colour is absent from a structure, owing to the absence of chromatin or through the stain having been washed out.

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[1] "A Critical Review of the Relation of Blood-sucking Invertebrates to the Life Cycles of the Trypanosomes of Vertebrates, etc.," by Captain Patton and C. Strickland. 'Parasitology,' vol. i, No. 4, Dec., 1908.

Fuchsin gave quite good nuclear pictures, but did not bring up the flagellar apparatus sufficiently well.

The drawings in the plate are made from two well infected leeches found infected in nature. They were at the early part of what I have called the middle period of digestion.

For convenience sake the periods of infection may be divided into three, corresponding to the stage of digestion:

(1) The early stage, when the blood is just coagulating, and when the first signs of the dark green-brown fluid is visible in the upper part of the intestine. The parasite at this time is in the condition of throwing off the original flagellum and adopting the resting state, during which division begins to take place. The parasites are for the most part still in the crop.

(2) Middle period of digestion, when the intestine is full of the green-brown fluid, where breaking down of the blood is going on actively. The parasite is now in the intestine in large numbers. It shows the whole range of forms, from the spherical non-motile creature to the typical Trypanosome. Great variation in size and thickness of the parasites is to be observed. Very slender, long forms are only occasionally to be seen. This middle period is of very long duration.

(3) Final period of digestion, when the crop is empty (or almost so) of blood, and the intestine nearly, or completely, free from the characteristic green-brown fluid. The Trypanosomes are now long, slender forms, with the kinetonucleus in the typical Trypanosome position. The forms now begin to remount the crop, and are also to be found in a still more slender condition in the proboscis.

The drawings, being made from leeches in the earlier phase of period (2), do not show the long, slender Trypanosomes developed during period (3). This final stage of the Trypanosome is most striking; but it is, of course, a matter of chance to get a leech in this condition in nature. This year I only got one; it was only slightly infected, and I was unfortunate in not getting fixed films.

It will be convenient first to give an account of the Trypanosome phase as found in the intestine of the leech (figs. 8—11), and then to give any points of interest in its development from the resting form.

The protoplasm is finely and evenly granular without vacuoles; indications of alveolar structure can be detected in some specimens, but are not very conspicuous. Protoplasmic inclusions are only occasionally present.

The trophonucleus is composed of a large central karyosome surrounded by a wide halo, which is in turn surrounded by a "membrane." Fine, but perfectly distinct, rays pass from the karyosome to the outer membrane. The karyosome is quite obviously made up of two substances, namely, the chromatin and an achromatic substance, in which the chromatin lies embedded. This achromatic substance frequently receives the name of plastin, and, while this does not convey any very clear idea, it is nevertheless a convenient and useful term. In Delafield's preparations the plastin stains a pale greyish-blue, in iron hæmatoxylin it is brownish, and it takes the green colour in the Twort's combination. The nature of the rays is a little obscure: they stain, as a rule, rather faintly with Delafield, but in some cases take the colour more deeply; Heidenhain shows them up black, but they wash out easily. I am inclined to regard them as more of the nature of plastin, but they seem at times to carry chromatin. The membrane shows very often little condensations of chromatin-staining material at the points where the rays meet it. The membrane stains well with Delafield and also with fuchsin, likewise with Heidenhain, but washes out long before the karyosome. The condensations on the membrane appear to me to be chromatin, but in Twort's stain they do not take up the red colour. I do not lay very much stress on this point, as it is just in a question of this kind that I think such a stain as Twort's is rather unreliable. There seems to be in the membrane, as in other parts of the nucleus, an underlying substance of an achromatic nature, in or on which the chromatin is deposited.

This nucleus is exceedingly constant in all the stages of the parasite as found in the leech, the only variation lying in slight differences in the condensation of the chromatin in the karyosome and the membrane.

The pictures presented in the dried Giemsa preparations differ greatly from this account. The most curious feature about this is that some of the Giemsa appearances give a very tolerably accurate representation, while others depart completely from the type shown by the wet method. The eight chromosomes so often seen in the Giemsa nuclei are not to be detected in the hæmatoxylin films. The rays and the condensations on the membrane are, I have no doubt, the manner in which these appear in the wet films. The number of the rays can, however, not be made out, as they are excessively fine, nor do the condensations on the membrane stand out sufficiently separately to be considered as individual structures. The Giemsa stain, of course, always increases the apparent size of any nuclear element into which it penetrates.

The kinetonucleus takes all the stains mentioned with great intensity; it is relatively large and rod-shaped. In close proximity, and apparently attached to it, lies the blepharoplast (Minchin, 'Quart. Journ. Micr. Sci.,' May, 1908, vol. 52), (figs. 9–10, etc.).

This structure will be more fully considered when the development of the flagellum is discussed. The blepharoplast stains with iron hæmatoxylin, but the stain is washed out more readily than from the nuclear structures; it appears grey-blue and rather faint with Delafield, and is difficult to detect at all in Twort preparations. The flagellum runs forward from the blepharoplast, ending, as usual, in a free whip. It stains green with Twort's combination and faintly with Delafield—more deeply with iron hæmatoxylin. The undulating membrane is developed to a varying degree, but is never much frilled.

Two other structures remain to be described. The first is a small granule only staining in Heidenhain preparations. It lies posterior to the kinetonucleus and often near the posterior end of the body. Sometimes it appears to be connected to it by a delicate strand. This granule is also found in the Giemsa preparations and is clearest in the Trypanosome phase. It has possibly something to do with the anchoring of the kinetonucleus (figs. 9 and 10).

The second is an element which I have never seen in the dried preparations at all, but which is a pretty constant feature in the wet films and appears equally so with all the four stains used. Just anterior to the trophonucleus a small condensation is to be observed in the protoplasm surrounded by quite a definite little halo. In rather dark Heidenhain preparations it stains almost black (figs. 9 and 10), with Delafield it looks grey-blue with soft outlines, and is usually only very slightly darker than the surrounding protoplasm (figs. 8, 11 and 19). It stands out clearly in these films more by reason of the halo than on account of its greater depth of colour. In Fuchsine films it is very clear: it is also visible in Twort's preparations but does not take on the red colour. It sometimes appears to be double. The nature and function of this structure is quite obscure: it is most clearly visible in the Trypanosome stage and its position is quite constant, but it is also present in the earlier phases. The position of the kinetonucleus often obscures it in these and makes it difficult to see.

The Trypanosome just described arises, as has been said, from a rounded resting form which develops a flagellum (figs. 1—11). The body gradually elongates and the kinetonucleus migrates backwards until it is well posterior to the trophonucleus. An undulating membrane develops during this process and the creature takes on the typical Trypanosome facies. The only point about this that calls for special attention is the development of the flagellum.

It may be noted in passing that, owing to the flattening of the Trypanosomes prepared by the dry method, certain details may be more distinctly visible in such specimens than in those prepared by the wet method.

The earliest stage of the development of the flagellum, of which one can be quite certain, is shown in fig. 2. Here it will be seen that two little projections have grown out from the kinetonucleus which is itself in process of division. These little structures sometimes take the Heidenhain rather deeply; they are not, however, very easy to make out as any obliquity in the position of the kinetonucleus is apt to obscure them. Later stages are shown in figs. 3—5. Here the flagellum appears as a thick strand arising from a granule with not very definite contours, which is in turn attached to the kinetonucleus. This granule at the origin of the flagellum is the blepharoplast. The minute detail is not very clear in the wet preparations, but as far as I can make out the blepharoblast seems to be attached to the kinetonucleus by a double thread. This may be seen in much later stages (figs. 5 and 6). The blepharoplast seems like the kinetonucleus to be ultimately a thin rod-shaped body. It seems to arise from the kinetonucleus, but I do not think in the light of its behaviour with the various stains that it is a chromatic body. It is true it often stains a deep sharp black with iron-hæmatoxylin, but that is no test for chromatin. It takes on a grey-blue pale colour with Delafield and shows up only dimly when at all with Fuchsin. In Twort's combination it stains green like the flagellum and is not very clear or precise. I am therefore inclined to regard the blepharoplast and flagellar apparatus which grows out from it as achromatic. Our knowledge of achromatic nuclear elements is so limited at present that it is impossible to say whether they can be regarded as an expression of the achromatic elements of the kinetonucleus or not.

Giemsa's stain presents a greatly exaggerated picture of the above development. The drying pulls the blepharoplast away from the kinetonucleus and makes the thread joining them stand out very clearly; it also greatly enlarges the apparent size of the blepharoplast, and very markedly increases the flagellar rudiments, which are always thick at this stage. In addition to all this these flagellar structures stain a deep red corresponding to the chromatic colour. That such a stain should be a fruitful source of error is obvious. It must, however, in justice be said of this stain that it has done brilliant if unequal service to the advancement of our knowledge of blood parasites, and has, therefore, amply served its purpose.

The longitudinal division of the kinetonucleus which I held to be the first stage in the development of the flagellum has been seen again in resting forms (fig. 1), but in the light of the above observations I do not feel confident as to how this appearance should be interpreted. It is in these, as in the other specimens of Pontobdella previously examined, a rare appearance, and the evidence for considering this as the initial step in the formation of the flagellum is very much less clear in the wet films than in Giemsa preparations. Moreover, I have recently found in T. vittatæ that the longitudinal division of the kinetonucleus is the ordinary method instead of the transverse, as in T. raiæ; it is, therefore, possible that the point may be open to some variation.

Division.—The figs. 7, 12–17 give pictures of various division stages, and it is perhaps in this point that Giemsa's method has, generally speaking, been the least misleading. The division starts as a rule by the transverse division of the elongated kinetonucleus, but this point is open to slight variation.

The trophonucleus shows first an arranging of the chromatin in two masses within the karyosome (figs. 7 and 12). A well-developed spindle subsequently arises, but the chromatin is divided without the formation of an equatorial plate. The wet method brings up the spindle very well (figs. 13—17). Centrosomal functions of some kind seem exercised by the condensations at the extreme points of the spindle, and the chromatin seems to pass along the "fibres"[1] from the two main central masses in such a stage, as fig. 13, to either poles of the spindle. There is at a slightly later stage (figs. 14 and 15), often a curious double appearance in the nuclei. Figs. 16 and 17 give the final stages. The spindle persists for some time after the nuclei are reformed, and can be clearly seen as a bright line in the live specimens.

Division occurs at all the different stages of development. There is an occasional not very well defined tendency to multiple division. Equal division is the rule, but unequal division is sometimes met with. Considerable variation in the division of the protoplasm is seen, as shown in figs. 18—24. It will be observed that in figs. 20—22 the division of the protoplasm has started at the posterior end instead of the anterior, as is more usual. Specimens of this type were watched for many hours in the live state in the hope that they might be individuals conjugating, but no evidence in favour of this was forthcoming, and I was led to believe that they are in all probability merely cases of division.

Some very curious appearances were observed where the protoplasm had split into several rod-like processes. This is shown in fig. 24, and, while not a common appearance, is still far too frequently seen to be dismissed as a casual abnormality. The figures, I may say, hardly do justice to all the varying sizes and shapes to be seen at this stage in the development.

As I have already said in a recent paper[2] on T. vittatæ from the soft tortoise Emyda vittata, we seem to have in these forms a type of life-history which is probably very widespread. The rounding off process and the subsequent development of a flagellate stage occurs, as has been recently shown in many of the Trypanosomes from cold-blooded hosts.

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[1] I use the word fibre quite without prejudice. The word conveniently expresses the optical effect, and I have no means of knowing what they actually represent.

[2] 'Quart. Journ. Micr. Sci.,' vol. 53, part 4.

It seems to occur in the Trypanosome from Hyla arborea (França, 'Bull. Soc. Portug. Sc. Nat.,' 1907), also in T. granulosum (França), also in T. loricatum (Dutton, Todd, and Toby, 'Ann. of Trop. Med. and Parasit.,' No. 3, 1907).

The similarity between the life-history of T. vittatæ and T. raiæ hardly needs emphasising. T. raiæ, of course, shows clearly the adaptation to the peculiar habits of the Pontobdella, its relatively slow development and the long persistence of the resting phase being correlated with the stiffening of the blood in the crop and the very long period which the Trypanosome must pass in the leech owing to the extreme slowness of the digestion.

The work here recorded was carried out partly at the Millport Marine Station, partly in the Zoological Laboratories at Glasgow University and the Lister Institute, Chelsea.

Lister Institute;
April, 1909.

EXPLANATION OF PLATE 9,

Illustrating Miss Muriel Robertson's paper on "Further Notes on a Trypanosome found in the Alimentary Tract of Pontobdella muricata."

Figs. 1—24 are drawn with 2 mm. aprochr. immersion lens by Ziess, 1·40 N.A. long tube and oc. No. 12, with the assistance of the camera lucida. The magnification is approximately 4500 diameters.

Fig. 25 is drawn with the No. 2 eyepiece; the magnification is approximately 1600 diameters.

Figs. 1, 2, 3, 4, 8, 9, 10, 13, 15, 17, 18, 19, 21 and 23 are from Heidenhain's hæmatox. preparations. The remaining figures are from Delafield preparations.

All the figures, with the exception of 25, are from the leech Pontobdella.

Fig. 1.—Resting phase, showing longitudinal division of the kinetonucleus.

Fig. 2.—Early stage in development of flagellum.

Figs. 3, 4, and 5.—Stages showing newly-formed flagellum.

Figs. 6 and 7.—Crithidial stages.

Fig. 8.—Trypanosome phase.

Fig. 9.—Trypanosome showing blepharoplast, granule at posterior end, and the structure just anterior to the trophonucleus.

Figs. 10 and 11.—Trypanosome phase.

Fig. 12.—Early division phase. Note condition of trophonucleus, kinetonucleus and blepharoplast.

Fig. 13.—Division stage showing spindle.

Fig. 14.—Later division stage.

Fig. 15.—Division stage showing trophonucleus spindle and also second division of the kinetonucleus. This is a rather unusual appearance.

Figs. 16 and 17.—Later division stages.

Fig. 18.—Division of Trypanosome.

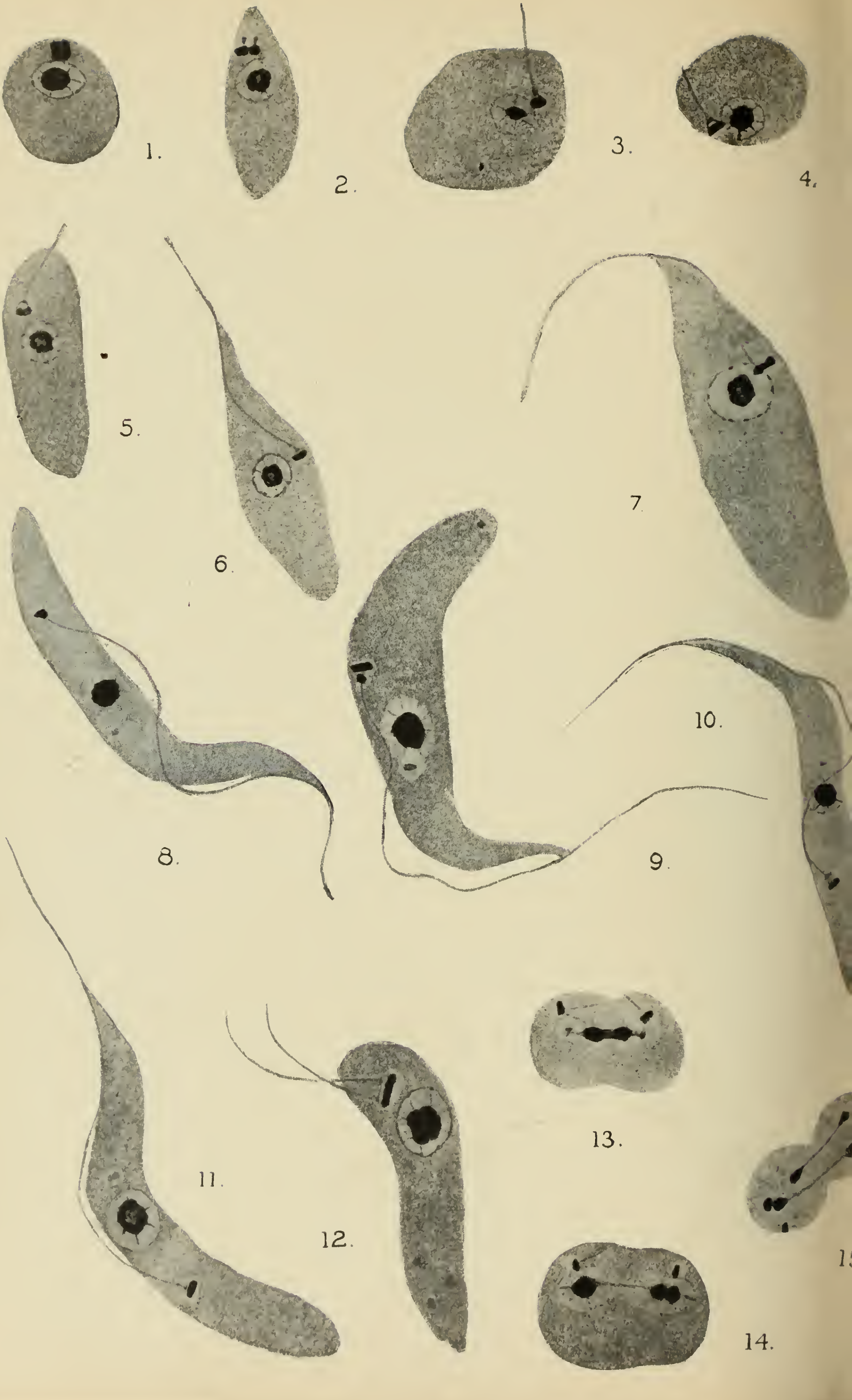
Fig. 19.—Division of Trypanosome.

Figs. 20–22.—Division stages where the protoplasm divides from the posterior end.

Fig. 23.—Rather unusual appearance where the posterior part of a dividing Trypanosome has formed a large rounded mass.

Fig. 24.—Division stage showing irregular splitting of the protoplasm.

Fig. 25.—T. raiæ from the skate's blood. Note this is drawn at a much smaller magnification (× 1600) than remaining figures.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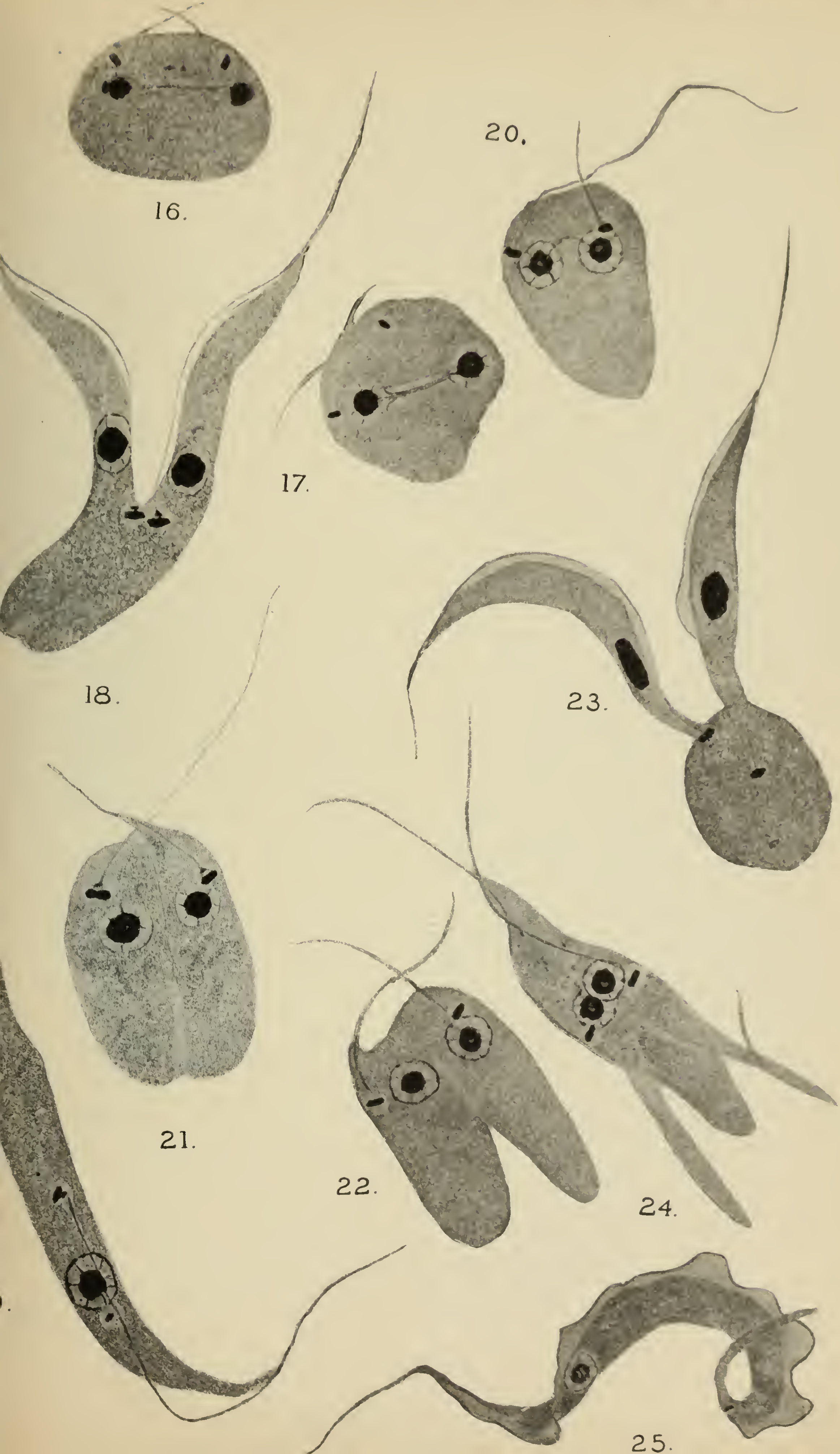
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The Development of the Parasite of Oriental Sore in Cultures.

By

R. Row, M.D.Lond., D.Sc.Lond.

With Plate 20.

[The following memoir is made up from two communications, dated respectively December, 1908, and January, 1909, which I received from Dr. Row. Each letter was accompanied by sketches and stained preparations; from the latter the figures on Pl. 20 have been drawn by my assistant, Miss Rhodes, at the Lister Institute. The account given here is, for the most part, in Dr. Row's own words; any remarks of mine are in square brackets.

The parasites causing oriental sore were first described accurately by Wright ('Journ. Med. Research,' vol. x, 1903, p. 472), and named by him Helcosoma tropicum. On account of their obvious affinity (which the present memoir confirms) with the Leishman-Donovan bodies of kala-azar, Wright's bodies have been referred by subsequent writers (compare Lühe in Mense's 'Handbuch der Tropenkrankheiten,' iii, 2, 1906, p. 203) to the previously established genus Leishmania Ross ('Brit. Med. Journ.,' 1903, vol. i, pp. 1261 and 1401), and they now stand as Leishmania tropica (Wright), the only other known species of the genus being L. donovani (Lav. et. Mesn.), the parasite of kala-azar.

It was first discovered by Rogers ('Quart. Journ. Micr. Sci.,' vol. 48, 1904, p. 367), and confirmed by many subsequent observers, that L. donovani gives rise in cultures to a flagellate Herpetomonas-like form. So far as I am aware no similar observations have been made for L. tropica, and the present memoir is the first account given of the cultural development of Wright's bodies. As will be seen, the result is very similar to that obtained for the Leishman-Donovan body. It is of some interest, however, that the method of cultivation required appears to be quite different in the two cases, a fact which indicates that the transmission and mode of development are different in the two parasites.

E. A. MINCHIN,
Rovigno, March, 1909.]

(1) Method of cultivation.—Material from the sore was planted in sterile sodium citrate solution (2 per cent.) and in blood-serum (human). Some of the cultures were left at the laboratory temperature (25°–28° C.), and some were incubated at 35° C.

Those incubated at 35° C. and those planted in the sodium citrate solution did not show any growth; on the contrary, after twenty-four, forty-eight, and seventy-two hours they seemed to have disintegrated, so that not even a trace of the original parasites could be seen. In one case (a sodium citrate culture at room-temperature) large staphylococcus-like bodies were seen; they were probably contaminations, and were not investigated further.

On the other hand, in the cultures in blood-serum at laboratory-temperature the parasites went through the development described in detail below; they increase in size, multiply greatly, and finally become Herpetomonas-like flagellates.

(2) The parasites in the sore.—In smears from the juice of the sore stained with Giemsa's stain the parasites are seen in all sorts of shapes—pear-shaped, oval, torpedo-shaped, and even spherical (figs. 1, 2, *a–f*, 3, *a–g*). They are found free outside the corpuscles and also in the large macrophages, in some of which they may occur in considerable numbers (fig. 1). The individual parasites consist of faintly staining protoplasm with macronucleus and micronucleus in various forms. The parasites appear to have a distinct capsule,

shown as a well-defined margin. The length of the body is about one-third that of a red blood-cell; the parasites are therefore a little smaller than those described by James (2), but on the whole the descriptions of Wright (3) and James are applicable to the bodies seen in my specimens.

The micronucleus seems to be more often dot-shaped than rod-shaped, and separation of the dot-shaped micronucleus from the macronucleus can be seen in different stages (figs. 2 and 3). From the study of the smears I am inclined to think that the youngest stage of the parasite is the torpedo-shaped form (fig. 2, *a–c*) with a single nucleus [or with the two nuclei closely apposed], and that the separation of the two nuclei in these spore-like forms comes about after the parasite has entered the macrophage; in other words, that the parasite undergoes development in the macrophage, and that the free parasites showing the typical characters of the two nuclei (fig. 2, *d*, *e*, fig. 3, *d*, *f*) are those which have been liberated from a macrophage, either by its disintegration or by ejection from it.

The parasites contained in macrophages are seen to be lodged inside a vacuole or clear space (fig. 1); possibly some sort of secretion is thrown out round the parasite by the macrophage.

The parasites multiply by fission (fig. 3, *e*, *g*), as described by previous observers. Heart-shaped nuclei are seen in some of the parasites in the smears.

(3) The phases of the development of the parasite in cultures.—After twenty-four hours the cultures, examined in the fresh condition or in stained smears, showed no very obvious increase in the numbers of the parasites, but a sparing distribution in groups of four or eight. The body of the parasite is increased to double its former size, but shows no other difference, except, perhaps, rather better staining of the body-protoplasm.

After forty-eight hours the parasites have grown and multiplied enormously, and are found in masses (fig. 4), visible under the low power. Isolated individuals are also to be found with high powers, but these are obviously separated from the colonies in the preparation of the smear. The masses consist of great numbers of parasites growing in colonies and entwined with one another. The colonies may be compact and more or less spherical, in which case it is more difficult to make out the shape of the individual parasites; or they may be looser in texture with the individuals more separate (fig. 4).

The parasites have now increased in length to two and a half or three times the diameter of a red blood-corpuscle, and in breadth to about half or two-thirds this diameter. The shape of each individual is like a banana (fig. 4); the contour of the body is well defined, the sides being parallel and the ends rounded; the anterior extremity bearing the micronucleus is more rounded than the other. The parasites are not mobile or amœboid, and for the most part no flagellum can be detected, though in some this organ is already present (fig. 5, *a–c*). The body-protoplasm shows a slightly spongy character, and takes up a deeper stain than in younger forms. The macronucleus is centrally situated and is less compact than in young forms. The micronucleus has shifted to one end of the body and shows a faint semblance of a vacuole round it.

At seventy-two hours the development is complete (fig. 6, *a–j*). The flagellum seems to be formed a few hours after the forty-eight-hour stage as an outgrowth from the micronucleus of a fine filament, which probably shoots out rapidly at the stage when the parasite may be supposed to have reached maturity. Once the flagellum is formed, the individual is liberated and is free to swim away from the colony. Here and there groups of six or more fully developed flagellated individuals are found, entangled by their intertwining flagella.

The body of the parasite is practically the same as described at forty-eight hours, except that it has grown a little longer and stouter, and the distinction between the posterior pointed and the anterior flagellate end is better marked. The flagellum is one and a half times or twice the length of the body of the parasite, and has from six to eight symmetrical undulations. In life the flagellum is very active, and moves very rapidly with a lashing, wave-like, not a corkscrew-like movement; in appearance it strongly resembles a spirochaete.

The parasites are frequently found in pairs (fig. 6, *f–j*) [doubtless indicating fission]. Sometimes a pair consists of a thinner and a thicker individual [compare the splitting off of spirillar forms in L. donovani, described by Leishman and Statham, 'Journ. R.A.M.C.,' iv, 1905, p. 321].

[The foregoing account contains the pith of Dr. Row's first letter to me, and appears to represent the normal healthy development of the parasite in cultures. With the object of obtaining a slower development of the parasite, especially of the stages previous to the formation of the flagellum, material was taken from a case of longer standing, and the parasites were allowed to "stew in their own juice" for three days in a sealed tube at laboratory temperature before cultivating them. The result was a slower development of the parasites in the cultures, with production of peculiar forms probably representing abnormal or degenerative forms of the parasites weakened by the unfavourable conditions of its development. An account of these cultures forms the substance of Dr. Row's second communication to me, and a brief abstract of it follows.—E. A. M.]

The parasite, when derived from an old case (in which the lesion is about to break down into pus and is on the point of ulcerating, and when, consequently, the contents of the lesion are rich in leucocytes and pus), gives rise in cultures to a slow and irregular development, both in numbers and in morphological characters. Under these unfavourable developmental conditions very few typical well-developed and mature flagellates are produced; the products of stunted forms either do not reach the flagellate stage at all, or, if they do so, they are unable to continue their existence long.

The following seems to be the general plan of the development. The parasites begin to elongate into ovoids (fig. 7, *a*) and then become pear-shaped (fig. 7, *b–h*), with one end pointed. The nucleus divides and division of the body follows (fig. 7, *j–l*). The pear-shaped parasite thus gives rise by fission to a slender form and a stout form (fig. 8, *a*). Each of these divides again twice (fig. 8, *b–i*), so that from one parasite are derived eight flagellates—four long forms and four short and stunted forms (compare fig. 8, *l*). The stunted forms are short-lived, but the long forms persist in cultures of even 120 hours' standing. All the division takes place before the flagellum is formed; after this event there seems to be no further multiplication. [This conclusion can only apply to the stunted cultures; in the more healthy cultures, as stated above, there is evidence of multiplication of the flagellated forms. Moreover, the vast masses of parasites in the forty-eight-hour healthy cultures indicate that here many more than eight flagellates are derived from a single Wright's body.]

ADDENDUM.

[When this memoir was completed and ready to send away I received, on March 27th, a third communication from Dr. Row, containing in concise form his conclusions, which I append here in his own words. Dr. Row exhibited his preparations and read a paper on them at the Medical Congress held in Bombay in February of this year.—E. A. M.]

I conclude that the parasite of the oriental sore (Leishmania tropica) and that of kala-azar (L. donovani), although apparently identical when examined in smears direct from the lesion, are distinct when examined in cultures, and for the following reasons:

(1) The parasite of oriental sore, when fully developed into the flagellate forms under ordinary conditions of culture, is much longer and bigger than that of kala-azar, where one meets with shorter and stouter forms, as a rule.

(2) The parasite of oriental sore is more resistant to external conditions than that of kala-azar; in other words it

is much less delicate, as it is possible to obtain developmental forms up to the flagellate stage from the parasite of oriental sore three days after its removal from the lesion, while the parasite of kala-azar, according to Rogers, dies within twenty-four hours after it leaves the spleen, if not cultured within that period.

(3) The flagellum of the parasite of oriental sore is much longer, and presents more regular wavy undulations than that of kala-azar, where it is shorter with less regular undulations.

(4) Although contamination of the material with extraneous germs is inhibitory to the early developmental progress of the parasite of oriental sore, it is not so destructive to its further development into flagellates as in the case of the parasite of kala-azar, where, according to Rogers, the slightest contamination with staphylococci is sufficient to destroy the culture.

(5) The parasite of oriental sore develops into fully mature flagellate forms between forty-eight and seventy-two hours, while that of kala-azar takes twice as long if not longer.

(6) The best culture medium (according to my results) for the parasite of oriental sore is human blood-serum, by preference that from tuberculous patients; while that for the parasite of kala-azar, according to Rogers, is sodium citrate, 2–10 per cent., in sodium chloride solution 0·8 per cent., mixed with splenic blood.

(7) The optimum temperature for the growth of the parasite of oriental sore is between 25° and 28° C., or even up to 30° C., while for the parasite of kala-azar it is 22° C., or even less, according to Rogers.

[Postscript (July 5th, 1909).—Since the above was sent to press I have been informed by Sir W. B. Leishman that the parasites of oriental sore have also been cultivated by Nicolle, whose memoir, however, I have not been able to see. —E.A.M.]

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2. JAMES, S. R.—"Oriental or Delhi Sore," op. cit., No. 13, 1905.

3. WRIGHT, J. H.—"Protozoa in a Case of Tropical Ulcer (Delhi Sore)" 'Journ. Med. Research, Boston,' x, 1903, p. 472, 4 pls.

EXPLANATION OF PLATE 20,

Illustrating Dr. Row's paper on "The Development of the Parasite of Oriental Sore in Cultures."

[All figures are drawn with the camera lucida to a magnification of 2000 diameters from preparations stained with Giemsa's stain.]

FIG. 1.—Large macrophage containing numerous parasites, from a smear of the juice of the sore.

FIG. 2, *a–f*.—Free parasites from the same smear; *a–c*, young forms with micronucleus and macronucleus closely apposed; *d–f*, parasites probably set free from a macrophage.

FIG. 3, *a–g*.—Free parasites from a smear of an old case, showing various shapes of the body and conditions of the nucleus; *e*, *g*, dividing forms.

FIG. 4.—Mass of parasites from a smear of a culture of forty-eight hours' standing, showing various shapes and sizes of the parasite.

FIG. 5, *a–c*.—Three flagellated individuals from the same preparation as the last figure.

FIG. 6, *a–i*.—Flagellated Herpetomonas-forms from a smear of a culture of seventy-two hours' standing; *a–e*, various forms of single flagellates; *f–i*, parasites in groups and pairs.

FIG. 7, *a–o*.—Parasites from a smear of a retarded culture of twenty-four hours' standing; various stages of the development are seen. Some parasites, such as the two small ones in *g*, have not developed at all; others, such as *k* and *l*, have developed much further and are dividing.

FIG. 8, *a–m*.—Parasites of a smear of a retarded culture of forty-eight hours standing. *a*, division into a stout and a slender form; *b–d*, division of stout forms; *e–i*, division of slender forms; *j*, *k*, groups showing various stages of development; in *j* is seen a parasite not advanced beyond the initial stage; *l*, a group showing a pair of slender forms, recently divided off, and a pair of stout forms in the act of dividing; *m*, two pairs of slender forms (one dividing) with flagella growing out.

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No 30

The Hexosephosphate formed by Yeast-juice from Hexose and Phosphate.

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It has been shown by Harden and Young* that—(1) the rate of fermentation of glucose by yeast-juice is greatly increased by the addition of a soluble phosphate; (2) this rate soon diminishes until a constant rate is attained, which is only slightly greater than that of the original yeast-juice and glucose; (3) during this period of increased fermentative activity, the phosphate undergoes some alteration, and at the end of the period is no longer present in a form precipitable by magnesium citrate mixture. The authors suggested in the first paper that a combination of the phosphate with the sugar, to form a phosphoric acid ester, had taken place, and more recently† embodied this suggestion in the form of an equation—

$$2C_6H_{12}O_6 + 2R'_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R'_2)_2 + 2H_2O.$$

It has also been shown that the same phenomena occur when fructose or mannose is used in place of glucose.‡

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* 'Chem. Soc. Proc.,' 1905, vol. 21, p. 189; 'Roy. Soc. Proc.,' B, 1906, vol. 77, p. 405.

† 'Roy. Soc. Proc.,' B, 1908, vol. 80, p. 299.

‡ 'Chem. Soc. Proc.,' 1908, vol. 24, p. 115; 'Roy. Soc. Proc.,' B, vol. 81, 1909.

In a paper on the changes that take place in the phosphorus compounds of plants, etc.,* Iwanoff stated that, during the fermentation of sugar by pressed yeast, the inorganic phosphates of the yeast are converted into organic compounds. This observation, however, remained unknown to the author of the present paper until the publication of a later paper by Iwanoff,† in which he described the formation of a phosphoric acid compound during the fermentation of glucose or fructose in the presence of sodium phosphate by yeast which had been previously treated with acetone ("zymin"). By precipitating with copper acetate, decomposing with sulphuretted hydrogen and evaporating, he obtained the free acids, corresponding to these compounds, in the form of dark brown syrups, which when prepared from glucose contained 19·6 to 20 per cent., and from fructose 13·4 to 14 per cent., of phosphorus. No other analyses were given in the paper. These syrups gave the characteristic reactions of aldo- and keto-groups, and yielded osazones when heated with phenylhydrazine, that from the glucose compound melting at 142°, and that from the fructose compound at 125°. Iwanoff concluded that these bodies were compounds of phosphoric acid either with a triose or with methylglyoxal, and that they were different according to the sugar from which they were derived.

At the time when Iwanoff's paper appeared, a considerable amount of work had been done by the author on this reaction, and a preliminary note was published describing the isolation of the compound by means of its lead salt.‡ It was shown that this salt had the empirical formula $C_3H_5O_2(PO_4Pb)$, and when decomposed with sulphuretted hydrogen yielded an acid which was slightly dextro-rotatory, and which, when the solution was boiled, underwent hydrolysis with formation of phosphoric acid and a lævorotatory reducing substance.

The present paper embodies the results of further work on this substance, and also on the similar compounds obtained by fermenting fructose and mannose in place of glucose. No difference has been detected between the compounds obtained from these three sugars.

Preparation of the Lead Compound.—A solution of 0·6 molar di-sodium or potassium hydrogen phosphate (R'_2HPO_4) was added to a mixture of yeast-juice and excess of glucose at 25°, in such an amount that rapid fermentation was set up. The quantity of phosphate which may be used varies with different juices,§ and the necessary amount was determined in each case in a small sample by measuring the rate of evolution of carbon dioxide under similar conditions. When the rate of fermentation had fallen, more phosphate was added, and the additions continued so long as this acceleration could be produced. The mixture was then boiled, and filtered from the coagulate formed.

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* 'Travaux de la Société des Naturalistes de St. Pétersbourg,' vol. 34, 1905.

† 'Zeit. physiol. Chem.,' 1907, vol. 50, p. 281.

‡ Young, 'Chem. Soc. Proc.,' 1907, vol. 23, p. 65.

§ Harden and Young, 'Roy. Soc. Proc.,' B, 1908, vol. 80, p. 299.

In a typical experiment, a mixture of 440 c.c. of yeast-juice and 55 grammes of glucose was employed, and 440 c.c. of the potassium phosphate solution (37 grammes, K_2HPO_4) were added 80 c.c. at a time. When fructose is used in place of glucose, much more phosphate may be added; thus in one case a mixture of 495 c.c. of yeast-juice and 200 grammes of fructose was employed, and 1000 c.c. of the phosphate solution (104·4 grammes K_2HPO_4) were added, 100 c.c. at a time.

The liquid, after boiling and filtering, was always found to contain a little free phosphate, and this was removed by adding a solution of magnesium nitrate, making alkaline with potash, stirring well, and allowing to stand for a few hours. The filtered liquid was then neutralised with acetic acid, lead hexosephosphate precipitated by the addition of lead acetate, and washed repeatedly with water by means of a centrifugal machine, until the washings no longer reduced Fehling's solution. It was found that this precipitate invariably contained traces of some nitrogenous material, and it was therefore suspended in water, decomposed with sulphuretted hydrogen, and the excess of this gas removed after filtering by passing a current of air through the solution. The liquid was then neutralised with potash and the lead salt reprecipitated with lead acetate, the process being repeated until the precipitate, after washing, was free from nitrogen. Usually two or three such precipitations were found sufficient. The salt was then filtered off and dried, first on a porous plate and then in a vacuum over sulphuric acid. It was thus obtained as a white, amorphous powder, free from nitrogen, which, on decomposing with sulphuretted hydrogen, yielded an acid solution containing no free phosphate precipitable by magnesium citrate mixture.

The preparation of yeast sold under the name of "zymin," which is obtained by treating pressed brewers' yeast with acetone, may be used in place of yeast-juice for the preparation of hexosephosphate. In one experiment 10 grammes of zymin, 10 grammes of glucose, and 100 c.c. of water were incubated at 25°, and 75 c.c. of a 0·3 molar solution of potassium phosphate added, 15 c.c. at a time. After rapid fermentation had ceased the mixture was filtered without boiling, and the lead compound obtained from the filtrate as before.

Lead compounds have been prepared from glucose, fructose, and mannose, and the analyses of several different preparations of these compounds are given in Table I. The mannose employed was obtained by the hydrolysis of ivory-nut, and was purified by being converted into the phenylhydrazone which was recrystallised from water and decomposed with benzaldehyde.

Table I.

No.	Origin of compound.	Weight taken.	CO_2.	H_2O.	$Mg_2P_2O_7$.	$PbSO_4$.	Percentages.			
							C.	H.	P.	Pb.
1	Glucose ...	0·5261	0·1870	0·0777	—	—	9·69	1·65	—	—
		0·6269	—	—	0·1775	0·5080	—	—	7·88	55·34
2	„ ...	0·4745	0·1696	0·0732	—	—	9·75	1·73	—	—
		0·5993	—	—	0·1704	0·4774	—	—	7·92	54·40
3	Fructose ...	0·3040	—	—	0·0890	0·2484	—	—	8·15	55·81
4	„ ...	0·4503	—	—	0·1337	0·3649	—	—	8·27	55·34
5	Mannose ...	0·3614	—	—	0·1021	0·2931	—	—	7·87	55·39
	$C_6H_{10}O_4(PO_4Pb)_2$ requires						9·60	1·33	8·27	55·20

Hexosephosphoric acid was obtained from the lead salt by suspending it in water, decomposing with sulphuretted hydrogen, and removing the excess of this gas from the filtered liquid by means of a current of air. An acid liquid was thus obtained which could be titrated with standard alkali, phenolphthalein being used as indicator, in a similar manner to phosphoric acid. In Table II several examples are given in which the amount of hexosephosphoric acid was estimated both by titrating the solution with decinormal alkali, and by decomposing with nitric and sulphuric acids, precipitating with magnesium citrate mixture, and weighing as magnesium pyrophosphate. The numbers all refer to 10 c.c. of the solutions.

Table II.

Origin.	Titration, c.c. N/10.	$Mg_2P_2O_7$.	Hexosephosphoric acid.	
			From titration.	From $Mg_2P_2O_7$.
Glucose	19·8	0·1086	0·1683	0·1664
„	63·4	0·3458	0·5390	0·5296
„	99·0	0·5434	0·8415	0·8323
„	9·6	0·0517	0·0816	0·0792
„	29·2	0·1559	0·2482	0·2388
Fructose	38·7	0·2124	0·3290	0·3253
Mannose	32·6	0·1802	0·2771	0·2761

When the solution was evaporated on the water-bath a charred mass was left which contained free phosphoric acid and had a strong odour of caramel, whilst even on evaporation under reduced pressure, or at ordinary temperatures in a vacuum over sulphuric acid, decomposition occurred and free phosphoric acid was formed. The solution was found to give Mohlisch's α-naphthol reaction. It reduced Fehling's solution only after some hours in the cold, rapidly on boiling: this reduction may be due to the hydrolysis of the compound with formation of a reducing hexose. This is known to take place, as an alkaline solution of the sodium salt that had been left standing for two or three days at room temperature was found to contain free phosphate, and a lævorotatory substance which reduced Fehling's solution in the cold in a few minutes. The same change was brought about more rapidly when the solution was boiled.

The acid was found to have a less reducing power when boiled with Pavy's ammoniacal copper solution than corresponded to an equivalent quantity of glucose. Thus five preparations from glucose gave reductions of 0·754, 0·788, 0·767, 0·732, and 0·800, an equivalent quantity of glucose being taken as 1. No osazone could be obtained, as on heating with phenylhydrazine acetate decomposition took place, and phenylhydrazine phosphate was formed. Attempts were also made to prepare insoluble hydrazones or osazones with phenylhydrazine, methylphenylhydrazine, benzylphenylhydrazine, parabromophenylhydrazine, and nitrophenylhydrazine, but in no case was any success attained.

No differences could be detected between the acids obtained from glucose, fructose, or mannose. They all yielded the same products on hydrolysis, formed salts having the same properties, and had approximately the same dextro-rotatory power. Table III gives the rotations of a number of preparations of these acids.

Table III.

No.	Origin.	Grammes per 10 c.c.	Observed rotation in 4-dm. tube. +	Temp.	α_D. +
1	Glucose	0·079	0·097	20°	3°·07
2	,, ...	0·539	0·671	18	3·11
3	,, ...	0·246	0·364	19	3·70
4	,, ...	0·114	0·161	16	3·53
5	,, ...	0·088	0·131	13	3·72
6	,, ...	0·178	0·278	16·5	3·90
7	,, ...	0·148	0·179	16	3·02
8	,, ...	0·247	0·333	15	3·38
9	,, ...	0·529	0·695	16·5	3·28
10	,, ...	0·425	0·560	25	3·29
11	Fructose	0·247	0·315	16·5	3·19
12	,, ...	0·314	0·416	18	3·31
13	,, ...	0·143	0·181	17	3·15
14	Mannose	0·152	0·189	19	3·12
15	,, ...	0·555	0·926	17	4·17
16	Yeast-juice	0·053	0·074	13	3·50

All these acids were prepared from the lead salts with sulphuretted hydrogen, excepting No. 13 which was obtained from the barium salt by means of dilute sulphuric acid. No. 16 was obtained from yeast-juice to which neither sugar nor phosphate had been added, whilst zymin was employed for the preparation of Nos. 6 and 11.

It will be observed that the differences between the values for the acids derived from fructose and mannose are of the same order as the differences among the glucose preparations themselves. With so low a rotatory power a small experimental error or a trace of optically active impurity would cause an appreciable difference in the α_D, and the differences seen in the table are probably due to these causes. The rotations of the barium salts of these acids, which are slightly soluble in cold water, were also determined, and were found to be approximately equal. These are given in Table IV.

Table IV.

Origin.	Grammes per 10 c.c.	Rotation in 4 dm. +	α_D 17°·5. +
Glucose	0 ·0665	0 ·085	3°·20
Fructose...............	0 ·0692	0 ·088	3 ·18
Mannose...............	0 ·0656	0 ·082	3 ·13

Hydrolysis of the Acid.

(a) *Production of Phosphoric Acid and a Lævorotatory Reducing Substance.*—When the solutions of the acid were boiled, phosphoric acid was gradually set free, the reducing power to Pavy's solution increased, and the solution became lævorotatory. Table V shows the rate at which the first two of these changes took place when a solution of hexosephosphoric acid prepared from glucose was boiled in a flask fitted with a reflux condenser.

The phosphoric acid is expressed as grammes of magnesium pyrophosphate

Table V.

Time, in hours.	Free phosphate.	Reduction.
0	0	0 ·258
2	0 ·167	0 ·297
4	0 ·238	0 ·342
7	0 ·304	0 ·371
11	0 ·365	—
15	0 ·401	0 ·373
27	0 ·467	0 ·371

and the reduction as the number of grammes of glucose to which it corresponded. The original solution contained 0·7167 gramme of the acid in 200 c.c. (approximately 0·01 molar), corresponding when completely hydrolysed to 0·511 gramme of magnesium pyrophosphate, and equivalent to 0·414 gramme of glucose. Samples were taken out after the intervals stated, and the free phosphate estimated with magnesium citrate mixture and the reducing power with Pavy's solution.

The solution after about seven hours' boiling became very dark coloured, some of the reducing substance formed evidently being decomposed.

The acids obtained from fructose and mannose behaved in a similar manner on boiling, in every case a lævorotatory substance and phosphoric acid were formed. The following Table VI gives a number of examples of this change in rotation on the hydrolysis, at 100°, of the compounds prepared from all three sugars and, in one case, No. 6, of that obtained from yeast-juice to which nothing had been added.

Table VI.

No.	Origin.	Time of boiling, in hours.	Rotation in 4-dm. tube.	
			Before. +	After boiling. −
			°	°
1	Glucose	6	0·161	0·299
		11	,,	0·558
		14	,,	0·658
2	,, ...	10	0·634	0·756
3	,, ...	6	0·543	0·924
4	Fructose	10	0·416	1·514
5	Mannose	6	0·230	0·204
6	Yeast-juice	16	0·074	0·500

As glucose is converted by alkalis into a lævorotatory mixture of glucose, mannose, and fructose, it was thought that the treatment with alkali in the separation of the free phosphate, during the preparation of the lead salt, might have altered the hexosephosphate in such a manner as to account for the lævorotatory sugar being obtained on hydrolysis. Preparations of the lead salt were therefore made without removing the free phosphate, the solution being kept always slightly acid, but the method being otherwise the same as before. The hexosephosphoric acids obtained from these acid preparations were found to have approximately the same specific rotatory power as the others. The strength of the solution used was estimated by determining the total phosphorus, and making allowance for the small quantity of free phosphoric acid, which was precipitated in a separate quantity with magnesium citrate mixture. In Table III, already given, Nos. 5, 6, 12, and 14 refer to acids prepared in this way, and it will be seen that they cannot be distinguished from those which had undergone the alkaline treatment. On hydrolysis, also, the same lævorotatory solution was obtained as is shown in Table VII.

Table VII.

No.	Origin.	Time of boiling, in hours.	Rotation in 4-dm. tube.	
			Before. +	After boiling. −
			°	°
1	Glucose	11	0·056	0·363
2	,, ...	8	0·131	0·370
3	,, ...	6	0·637	1·184
4	Fructose	2	0·317	0·276
	,, ...	4	,,	0·574
	,, ...	6	,,	0·956
	,, ...	8	,,	1·212

(b) *Nature of the Reducing Substance.*—To determine the nature of this reducing substance, the mixture from the hydrolysis of a preparation from glucose was exactly neutralised to litmus with barium hydrate, and the precipitation of the barium salts of phosphoric and unchanged hexosephosphoric acids completed by the addition of two volumes of absolute alcohol. After filtration, the solution was evaporated at 35° to 40° under reduced pressure to a thick syrup. This was found to reduce Fehling's solution in the cold, and to be lævorotatory. It gave the red coloration characteristic of fructose when heated with resorcinol and hydrochloric acid (Seliwanoff). When this syrup was treated at 0° with milk of lime, an insoluble calcium compound similar to calcium fructosate was formed. This was filtered off, washed with water, suspended in water, and decomposed with carbon dioxide, and the filtered liquid treated with animal charcoal and evaporated under reduced pressure.

The colourless syrup thus obtained reduced an alkaline solution of copper glycocolate after 12 hours in the cold, a reaction which, according to Pieraerts,* is only given by fructose. It readily formed an osazone when heated with phenylhydrazine in the usual manner, and this, after recrystallisation, first from alcohol and then from a mixture of pyridine and water, melted at the same temperature as glucosazone, viz., 206°, whilst a mixture of this compound with glucosazone melted at 206° to 207°. The analysis also corresponded to that of glucosazone—

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* 'Chem. Zentral.,' 1908, vol. 1, p. 1854.

0·1222 gramme gave 16·2 c.c. nitrogen at 16°·4 and 774·3 mm.
N = 15·76 per cent.

Glucosazone, $C_{18}H_{22}O_4N_4$, requires N = 15·64 per cent.

The fructose and mannose acids, when treated as above, yielded syrups having the same properties as that from the glucose acid; in both cases glucosazone was obtained from them.

All these syrups were found to induce rapid fermentation in a mixture of yeast-juice and glucose with such an excess of phosphate that fermentation was only proceeding at a very slow rate, and this induction has been shown to be brought about by the addition of fructose, but not of glucose or mannose.*

Finally, the ratio of the rotation to the reducing power was obtained and was found to be approximately the same as that found for pure fructose. The reducing power was determined by means of Pavy's ammoniacal copper solution and, as this was standardised, each time it was used, with a solution of pure glucose it has been found convenient to express the reduction per 100 c.c. of solution by the number of grammes of glucose which would reduce the same amount of copper, and to compare the rotation of the solution in a 4-dm. tube with this number. In Table VIII, this ratio, $\frac{\text{Rotation}}{\text{Reduction}}$, is given for the hexoses obtained by means of milk of lime from the products of hydrolysis of the acids derived from all three sugars, and it will be seen that it agrees in every case fairly well with that found for pure fructose.

Table VIII.

Sugar from—	−Rotation in 4 dm. tube.	Reduction as glucose per 100 c.c.	Ratio.
	°		
Glucose acid	1·806	0·512	3·52
Fructose acid (1)	0·962	0·284	3·38
,, (2)	0·516	0·151	3·41
Mannose acid	1·376	0·441	3·12
Pure fructose (1)	0·607	0·161	3·77
,, (2)	0·829	0·219	3·79

The solutions, after removal of the phosphate and hexosephosphate and before the treatment with milk of lime, always possessed a greater reducing power than a solution of fructose having the same optical activity. The ratio of the rotation to the reduction was always lower than that for pure fructose, and varied from 1·36 to 2·78. This might be accounted for by the presence of glucose or mannose, and experiments were made to ascertain if these sugars were present; in no case, however, could either be detected. The insoluble hydrazone of mannose was not obtained with phenylhydrazine, nor was a hydrazone formed when the syrup was treated with methylphenylhydrazine in the manner described by Neuberg* for the separation of glucose or mannose from fructose. They were also examined for glucose by the method by which Lobry de Bruyn and van Ekenstein† detected glucose in the mixtures obtained by the action of alkalis on fructose or mannose. This consisted in removing most of the fructose by precipitating as calcium fructosate or by extracting successively with alcohol, ethyl acetate, ether, and acetone, and identifying glucose in the residue either by converting into α-methyl glucoside by means of methyl alcoholic hydrochloric acid, or by oxidising with dilute nitric acid and precipitating the acid potassium salt of the saccharic acid formed. Neither of these compounds could be obtained from the syrups under examination.

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* Harden and Young, 'Chem. Soc. Proc.,' 1908, vol. 24, p. 115; 'Roy. Soc. Proc.,' B, 1909.

In order to ascertain whether these low ratios were due to the action of the phosphoric acid on the fructose after it was set free, a mixture of these compounds in the same relative proportions as would be contained in hexosephosphoric acid was boiled for some hours, and the phosphoric acid then removed by means of barium hydrate and alcohol in a similar manner to that described for the hydrolysed acid. The results of two experiments are given in Table IX.

Table IX.

Time, in hours.	−Rotation in 4 dm. tube.	Reduction per 100 c.c.	Ratio.
(1) 0	7·424	1·927	3·87
10	3·832	1·082	3·54
15	2·580	0·791	3·26
(2) 0	0·711	0·188	3·78
10	4·250	1·276	3·33

In both these experiments the solutions became very dark coloured and a considerable quantity of fructose had been destroyed. It will be observed that in both cases the ratio became less on boiling, but the lowest value obtained was much higher than the numbers found for the product from hexosephosphoric acid. It is possible that the fructose as it is being formed is much more susceptible to the action of phosphoric acid. On the other hand, these low ratios may be due to the presence of some other reducing substances having a less lævorotatory power than fructose.

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* 'Ber.,' 1902, vol. 35, p. 959.

† 'Rec. Trav. Chim.,' 1895, vol. 14, p. 156.

Salts of Hexosephosphoric Acid.

In addition to the lead salt already described, several other salts have been prepared. Attempts to obtain the potassium and sodium salts were made by adding the hydrates to the acid solution until neutral to phenolphthalein, and evaporating the solution under reduced pressure. A sticky mass was left which would not crystallise, and which decomposed on keeping.

Solutions of the alkali salts slowly decomposed on keeping, free phosphate being formed and the solution becoming lævorotatory. This decomposition causes a small error in the estimation of the free phosphate present in a mixture of this salt and hexosephosphate by means of magnesium citrate mixture. Many of the salts are more soluble in cold than in hot water; thus, when magnesium citrate is added to a solution of sodium hexosephosphate, no precipitate is formed until the mixture is heated: a white precipitate then comes down, which redissolves on cooling. The manganese, barium, and calcium salts behave in a similar manner.

Silver Hexosephosphate, $C_6H_{10}O_4(PO_4Ag_2)_2$.—This salt was obtained by neutralising the free acid with caustic soda, adding the calculated quantity of silver nitrate and then half the total volume of alcohol. The precipitate was filtered off, well washed with a mixture of water and alcohol, dehydrated with alcohol and ether, and dried over sulphuric acid in a vacuum, and it was thus obtained as a white amorphous powder. It was exceedingly unstable, and the whole preparation was carried out by red light. Even when dry the salt darkened on exposure to light, whilst when heated with water it was at once reduced to metallic silver.

The following analyses were carried out with different preparations of this salt:—

Table X.

Origin.	Weight taken.	AgCl.	$Mg_2P_2O_7$.	Percentages.	
				Ag.	P.
Glucose (1) ……………	0 ·2340	0 ·1740	0 ·0644	55 ·94	7 ·67
„ (2) ……………	0 ·3976	0 ·2989	0 ·1057	56 ·54	7 ·40
Fructose (1) ……………	0 ·3293	0 ·2439	0 ·0893	55 ·75	7 ·55
„ (2) ……………	0 ·3214	0 ·2378	0 ·0909	55 ·70	7 ·87
$C_6H_{10}O_4(PO_4Ag_2)_2$ requires……………………				56 ·24	8 ·08

Barium hexosephosphate, $C_6H_{10}O_4(PO_4Ba)_2$, was obtained by adding a solution of barium chloride to a solution of potassium or sodium hexosephosphate, and warming the mixture on the water bath. A granular white precipitate was thus formed, which was found to be more soluble in cold than in hot water. This was purified by dissolving in cold water and reprecipitating by warming the solution. It was then filtered off, washed with warm water, and dried over sulphuric acid in a vacuum. At 17°·5 100 c.c. of solution were found to contain 0·665, 0·692, and 0·656 gramme of the barium salts derived from glucose, fructose, and mannose respectively. The following analyses were obtained for this salt:—

Table XI.

Origin.	Weight taken.	$BaSO_4$.	$Mg_2P_2O_7$.	Percentages.	
				Ba.	P.
Glucose (1)	0·2578	0·1994	0·0960	45·50	10·36
" (2)	0·5298	0·4002	—	44·46	—
	0·4317	—	0·1538	—	9·92
Fructose (1)	0·4009	0·3081	0·1485	45·23	10·32
" (2)	0·5221	0·3933	0·1902	44·34	10·14
Mannose	0·4186	0·3179	0·1538	44·70	10·22
$C_6H_{10}O_4(PO_4Ba)_2$ requires				44·92	10·16

Calcium Hexosephosphate.—A calcium salt of the acid derived from glucose was obtained by adding calcium chloride to a solution of the sodium salt, and completing the precipitation by adding alcohol. When dried over sulphuric acid in a vacuum it gave the following analysis:—

0·3488 gramme gave 0·1958 gramme CO_2, 0·0917 gramme H_2O,
0·0890 gramme CaO, and 0·1778 gramme $Mg_2P_2O_7$.

C = 16·81, H = 2·92, Ca = 18·24, P = 14·20 per cent.

$C_6H_{10}O_4(PO_4Ca)_2 + H_2O$ requires C = 16·59, H = 2·76, Ca = 18·41,
P = 14·28 per cent.

Source of the Hexosephosphate.—In order to show that this compound was formed from the added phosphate, two quantities of 50 c.c. of yeast-juice were incubated at 25°, one with 30 c.c. of a 30-per-cent. solution of glucose and the other with 30 c.c. of a 0·3 molar solution of potassium phosphate containing 30 per cent. glucose. After two hours both were boiled and filtered, 30 c.c. of each filtrate were treated with the same quantity of magnesium nitrate and potash to remove any free phosphate and, after standing for a few hours, the solutions were filtered, neutralised with acetic acid and lead hexosephosphate precipitated with lead acetate, washed and dried over sulphuric acid in a vacuum. The mixture to which the phosphate had been added gave 5·03 grammes of lead salt, whilst the other only yielded 1·35 grammes. This experiment also shows that yeast-juice itself contains a small quantity of hexosephosphate, and experiments have already been quoted to show that this compound has similar properties to that formed when phosphate and sugar are added. The same hexosephosphate was obtained by means of zymin in place of yeast-juice as already mentioned, and as in this case the mixture was not boiled before the precipitation of the lead salt, it follows that the salts of this acid were present in the mixture as such, and were not formed from a more complex body by the boiling, which was always necessary when yeast-juice was employed. A hexosephosphate has also been detected in the extract obtained by boiling pressed brewers' yeast with water.

Molecular Weight of Hexosephosphoric Acid.—The analyses of the salts of this acid gave no information as to its molecular weight, since the same percentage composition would be obtained if the acid had the formula $C_3H_5O_2(PO_4H_2)$ and not $C_6H_{10}O_4(PO_4H_2)_2$. The first compound might be expected on hydrolysis to yield glyceraldehyde or dioxyacetone, and these might polymerise to form hexoses; so that the fact that fructose was formed when the acid was hydrolysed does not do away with the possibility of the smaller formula.

As no derivatives of the acid could be obtained, such as a hydrazone or an osazone, analysis of which would have settled the question, recourse was made to physical methods. The molecular weight of the acid was calculated from the difference between the freezing point of its solution and that of water, and was compared with that calculated by taking into consideration the extent to which it was dissociated in solution as determined by the rate at which it hydrolysed cane sugar. The freezing points of three solutions of different concentrations of the acid prepared from glucose was found by the ordinary method, the amount of acid in solution was estimated by titration and checked by the total phosphorus content, and the weight of water in the solution was obtained from the specific gravity. By way of comparison with a compound of known constitution and of a similar nature, corresponding determinations were carried out with a sample of synthetical glycerophosphoric acid, which was purified by means of its lead salt in exactly the same manner as the hexosephosphoric acid. The results are given in Table XII, Nos. 1, 2, and 3 being those obtained from the hexosephosphoric acid, and Nos. 4 and 5 from two concentrations of glycerophosphoric acid.

Table XII.

No.	Normality.	Grammes per 100 c.c.	Specific gravity.	Weight of water.	Depression.	Molecular weight.
1	1·75	15·13	1·075	92·37	1·747°	177
2	0·99	8·42	1·042	95·78	0·932	178
3	0·55	4·68	1·022	97·52	0·535	169·4
4	1·65	14·21	1·063	92·09	2·153	135·5
5	0·97	8·38	1·037	95·32	1·300	127·7

The formula $C_6H_{10}O_4(PO_4H_2)_2$ corresponds to a molecular weight of 340, and the smaller formula to 170, whilst glycerophosphoric acid, $C_3H_7O_2(PO_4H_2)$, has a molecular weight of 172. Thus the value obtained, although low, was much greater than that found for glycerophosphoric acid, which only differs in composition from the smaller formula by two hydrogen atoms.

The cane-sugar hydrolysis was carried out at 25° with semi-normal acid, and the rate was compared with that found for semi-normal hydrochloric and glycerophosphoric acids under similar conditions.

Table XIII gives the figures found for the hexosephosphoric acid and Table XIV those for the glycerophosphoric acid. Allowance has been made in the tables for the rotation due to the acids themselves. Solutions of the hexosephosphoric and glycerophosphoric acids of the same normality were also kept by themselves under similar conditions for the same time, and no alteration took place in their rotations.

Table XIII.—Hexosephosphoric Acid.

Time, in minutes.	Rotation.	x.	$A-x$.	$K \times 10^4$ $\left(K = \frac{1}{t} \log \frac{A}{A-x}\right)$.
0	+26·83°	0	34·88	—
30	25·95	0·88	34·00	3·700
60	24·98	1·85	33·03	3·945
90	24·01	2·82	32·06	4·069
120	23·12	3·71	31·17	4·070
160	22·05	4·78	30·10	4·001
180	21·44	5·39	29·47	4·051
210	20·66	6·17	28·71	4·026
240	19·95	6·88	28·00	3·976
310	18·17	8·66	26·22	3·999
330	17·72	9·11	25·77	3·984
360	17·06	9·77	25·11	3·965
390	16·32	10·51	24·37	3·993
420	15·70	11·13	23·75	3·974
∞	−8·05	34·88		
			Mean.........	3·981

Table XIV.—Glycerophosphoric Acid.

Time, in minutes.	Rotation.	x.	$A-x$.	$K \times 10^4$.
0	+26·36	0	34·41	—
31	25·51	0·85	33·56	3·503
60	24·79	1·57	32·84	3·380
90	24·06	2·30	32·11	3·338
120	23·30	3·06	31·35	3·370
150	22·64	3·72	30·69	3·312
182	21·86	4·50	29·91	3·344
210	21·23	5·13	29·28	3·339
240	20·54	5·82	28·59	3·353
270	19·89	6·47	27·94	3·350
300	19·26	7·10	27·31	3·345
330	18·64	7·72	26·69	3·343
360	18·01	8·36	26·05	3·358
∞	−8·05	34·41		
			Mean.........	3·353

Semi-normal hydrochloric acid under the same conditions gave the constant $K \times 10^4 = 22·466$.

It follows from these rates that the ratio of hydrogen ions in the solution of hexosephosphoric acid to those in the hydrochloric acid was 3·981 to 22·466 or 17·7 to 100, whilst in the case of glycerophosphoric acid the ratio was 3·353 to 22·466 = 14·9 to 100. As hydrochloric acid is almost completely dissociated, these numbers may be taken as a measure of the concentration of hydrogen ions in the solutions. Glycerophosphoric acid is dibasic and a solution which is semi-normal by titration will contain only half as many molecules as a semi-normal solution of hydrochloric acid, or 50 molecules for every 100 of hydrochloric acid. If the glycerophosphoric acid be assumed to dissociate into two ions, then out of every 50 molecules 14·9 are dissociated into $14·9 \times 2 = 29·8$ ions, and $50 - 14·9 = 35·1$ molecules remain undissociated. The solution will thus contain $29·8 + 35·1 = 64·9$ units, and the freezing point depression will correspond to a molecular weight of $\frac{50}{64·9} \times 172 = 132·5$; those actually observed gave 135·5 and 127·7. Similarly with the hexosephosphoric acid, if the larger formula obtain, the acid is tetrabasic and a semi-normal solution will only contain one-quarter as many molecules as a semi-normal solution of hydrochloric acid, or 25 molecules to every 100 of hydrochloric acid. If the dissociation take place into two ions, of these 25 molecules 17·7 are dissociated into $17·7 \times 2 = 35·4$ ions, $25 - 17·7 = 7·3$ molecules remain undissociated, and the total number of units will be $7·3 + 35·4 = 42·7$. The molecular weight of the acid from the freezing point of this solution will be $\frac{340 \times 25}{42·7} = 199$; the observed values were 177, 178, and 169·4.

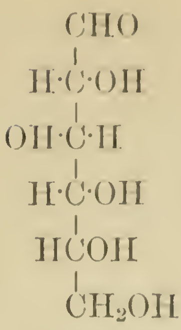
If, on the other hand, the compound have the smaller formula, with a molecular weight of 170, it will be a dibasic acid and the solution will contain 50 molecules to every 100 contained in a solution of hydrochloric acid of similar normality. The number of dissociated molecules corresponding to the hydrogen ions present will be 17·7, yielding $17 \cdot 7 \times 2 = 35 \cdot 4$ ions, $50 - 17 \cdot 7 = 32 \cdot 3$ will be undissociated, and the total number of units will be $35 \cdot 4 + 32 \cdot 3 = 67 \cdot 7$. The molecular weight, as deduced from the freezing point, would therefore be $\frac{170 \times 50}{67 \cdot 7} = 125 \cdot 5$.

The observed depression thus corresponds more closely to that which would be expected from the larger formula, although the agreement is not so good as that in the case of glycerophosphoric acid. This low result may be due to some hydrolysis in the solution, which would make the number of units still greater. If the hexosephosphoric acid had the smaller formula, it would be expected to depress the freezing point at least to the same extent as glycerophosphoric acid, since it only differs from this by two hydrogen atoms, and is slightly more dissociated in solution than this acid. These results thus point to the acid having the formula $C_6H_{10}O_4(PO_4H_2)_2$.

Further evidence against the acid being a derivative of glyceraldehyde is also afforded by the following facts:—When the acid was reduced with sodium amalgam, no glycerol was obtained, as might have been expected if it were a compound of glyceraldehyde. When glycerophosphoric acid was heated with hydriodic acid in a current of carbon dioxide, it was reduced to isopropyl iodide, which was detected by passing into alcoholic silver nitrate in the manner in which glycerol is estimated by the method of Zeisel and Fanto. On the other hand, hexosephosphoric acid gave no volatile iodide when treated in this manner.

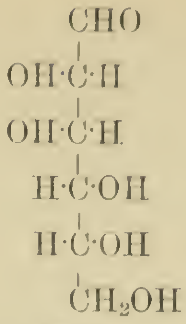
Constitution of Hexosephosphoric Acid.—It has not yet been found possible to obtain any evidence with regard to the position of the phosphoric acid groups in the molecule. The facts that the compound does not reduce Fehling's solution in the cold until after a long time, and that no hydrazones or osazones could be obtained, render it possible that the acid does not contain an active carbonyl group, and it may be that this group is involved in the reaction with the phosphoric acid.

Glucose, fructose, and mannose all appear to yield the same hexosephosphoric acid when fermented by yeast-juice in the presence of phosphate. One explanation of this is that these sugars are changed, before conversion into hexosephosphate, into some form common to all three. The only differences between glucose, fructose, and mannose are in the groups or arrangement of the groups attached to the first two carbon atoms:



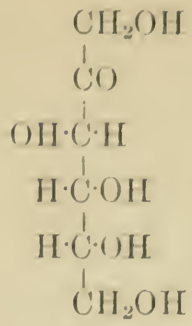
$$\begin{array}{c} CHO \\ | \\ H\cdot C\cdot OH \\ | \\ OH\cdot C\cdot H \\ | \\ H\cdot C\cdot OH \\ | \\ HCOH \\ | \\ CH_2OH \end{array}$$

Glucose.



$$\begin{array}{c} CHO \\ | \\ OH\cdot C\cdot H \\ | \\ OH\cdot C\cdot H \\ | \\ H\cdot C\cdot OH \\ | \\ H\cdot C\cdot OH \\ | \\ CH_2OH \end{array}$$

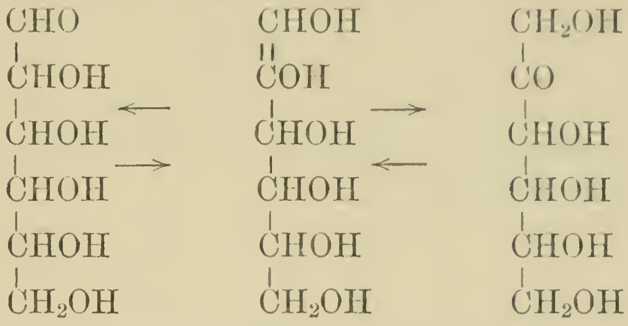
Mannose.



$$\begin{array}{c} CH_2OH \\ | \\ CO \\ | \\ OH\cdot C\cdot H \\ | \\ H\cdot C\cdot OH \\ | \\ H\cdot C\cdot OH \\ | \\ CH_2OH \end{array}$$

Fructose

and all these sugars have the same enolic form:



$$\begin{array}{ccccc} CHO & & CHOH & & CH_2OH \\ | & & \| & & | \\ CHOH & & COH & & CO \\ | & \longleftarrow & | & \longrightarrow & | \\ CHOH & & CHOH & & CHOH \\ | & \longrightarrow & | & \longleftarrow & | \\ CHOH & & CHOH & & CHOH \\ | & & | & & | \\ CHOH & & CHOH & & CHOH \\ | & & | & & | \\ CH_2OH & & CH_2OH & & CH_2OH \end{array}$$

Glucose and mannose. Enolic form. Fructose.

and are converted into one another in alkaline solution,* and it is conceivable that the hexosephosphate is a derivative of this enolic form.

If the reaction take place according to the equation:

$$2C_6H_{12}O_6 + 2R'_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R'_2)_2 + 2H_2O,$$

two molecules of sugar are concerned, and another possibility is that the phosphoric acid groups are attached to a new molecule, which is formed by the combination of portions of each of these two sugar molecules. In this case, also, the hexosephosphoric acid might be expected to have the same composition when formed from different sugars.

Summary.—1. The compound formed during the accelerated fermentation of glucose, fructose, and mannose by yeast-juice, in the presence of a soluble phosphate, is a salt of an acid which probably has the formula $C_6H_{10}O_4(PO_4H_2)_2$, and may be isolated by precipitation of its lead salt.

2. The free acid may be obtained in solution by decomposing this lead salt with sulphuretted hydrogen.

3. The acid is very unstable and readily decomposes on keeping, or on evaporating even at ordinary temperatures in a vacuum over sulphuric acid, with formation of a reducing substance and phosphoric acid.

4. It reduces Fehling's solution only after some hours in the cold, rapidly on boiling, whilst no osazones or hydrazones have been obtained from it.

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* Lobry de Bruyn, 'Rec. Trav. Chim.,' 1895, vol. 14, p. 201.

5. No differences have been detected between the hexosephosphoric acids or their salts, whether derived from glucose, fructose, or mannose.

6. On hydrolysis of the acid by boiling, phosphoric acid is set free and fructose formed. No other hexose could be identified, but the solution, after hydrolysis, was always less lævorotatory than a solution of pure fructose of the same reducing power.

7. The salts of lead, barium, silver, and calcium have been prepared.

[The compound containing phosphorus, which was considered to be phenylhydrazine phosphate, has since been examined by von Lebedew (‘Biochem. Zeits.,’ 1909, vol. 20, p. 113), who regards it as a phenyl hydrazido-phosphoric acid compound of hexosazone. A re-examination of this substance by the author leads to the conclusion that it is in reality a derivative of hexosephosphoric acid, but decisive results as to its constitution have not yet been obtained.—*November* 15, 1909.]

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